

Lipoic Acid and its Potential Role in the Treatment of Oxidative-Induced Injury of A549 Lung Epithelial Cells

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Abstract

Oxidative stress is the imbalance between prooxidants and antioxidants within a cell that can cause and/or contribute to the progression of many pathological conditions. Reactive oxygen species (ROS) formed from the metabolism of hydrogen peroxide (H_2O_2) or paraquat (PQ) can damage DNA, proteins, and polyunsaturated fatty acids (PUFAs). A cell's ability to defend against oxidative stress depends on multiple factors including genetic make-up, subcellular organelle function and environmental factors. Lipoic acid (LA) is an antioxidant with high reactivity to free radicals, but because of its low redox potential, it is also known to regenerate ascorbic acid and α -tocopherol, elevate intracellular glutathione (GSH) levels and modulate transcription factor activity. We used A549 lung epithelial cells in an *in vitro* model to study the mechanism(s) of LA effects against H_2O_2 - and PQ-induced oxidative injury. We observed that LA was incapable of protecting against H_2O_2 toxicity; thus, we used PQ as the agent to induce oxidant-induced damage. Challenge of cells with PQ resulted in decreases of cell viability and mitochondrial membrane potential and increases in DNA fragmentation and intracellular Ca^{2+} levels. Challenge of cells with LA resulted in an increase in cell viability, but a decrease in mitochondrial membrane potential. Interestingly, LA pretreatment of PQ-challenged cells resulted in the potentiation of DNA fragmentation and intracellular Ca^{2+} levels. Our results indicate that LA enhanced the PQ-induced oxidative injury in A549 lung epithelial cells, perhaps by acting as a prooxidant.

Lay Summary

Oxidative stress is an exaggeration of normal biochemical events within the human body. About 4% of oxygen molecules, under healthy conditions, form free radicals that cause damage to cell membranes, proteins and DNA. Our bodies have antioxidant defense systems that neutralize free radicals, unless it's overwhelmed by excessive oxidant stimuli. Paraquat (PQ) is a non-selective herbicide currently used in over 100 countries, including Canada and the United States. Oxidative injury caused by PQ is primarily localized in the lung where it's selectively taken up and undergoes oxidation-reduction reactions to form reactive oxygen species. Lipoic acid (LA) is an antioxidant with high reactivity toward reactive oxygen species and the ability to improve other antioxidant systems. Our research goal was to determine whether LA could protect against PQ-induced oxidative stress in lung epithelial cells. Although LA seems to be an ideal antioxidant, results from our studies showed that LA did not protect against oxidant-induced injury reminding us how the form doesn't always fit the function.

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Introduction

1.1 Oxidative Stress:

Oxidative stress is the imbalance between prooxidants and antioxidants that can lead to the etiopathogenesis and/or progression of many pathological states such as diabetes (West, 2000), neurodegenerative diseases (Barnham *et al.*, 2004), and tumour formation (Weinberg, 2007; Bilska *et al.*, 2005). As part of normal physiology as well as in response to external challenges posed by various microorganisms and chemicals, biological systems continuously generate reactive oxygen species (ROS) to fend off these agents but in turn they make themselves vulnerable to the detrimental effects of ROS (Rahman *et al.*, 2006). These ROS may be endogenously produced by metabolic reactions such as mitochondrial electron transport during respiration and activation of phagocytes, or exogenously produced following exposure to air pollutants, cigarette smoke and radiation (Klaassen, 2001). A cell's ability to defend against oxidative stress depends on multiple factors including genetic make-up, subcellular organelle function and environmental factors such as diet, smoking and many others (Keaney, 2000). The antioxidant capability of a cell is ultimately determined by the supply of reducing equivalents (Kehrer *et al.*, 1993).

1.2 Reactive Oxygen Species:

Reactive oxygen species (ROS) are formed from normal physiological and metabolic processes that reduce oxygen. In the electron transport chain, cytochrome-c oxidase is used to catalyze the four-electron reduction of oxygen into water (Thannickal, 2000; Yu, 1994). During the electron transfer reactions other oxygen metabolites can be formed, which include the superoxide radical, hydrogen peroxide and the hydroxyl radical. The superoxide and hydroxyl radical are thermodynamically unstable because they lack a paired valence electron (Yu, 1994). Because of their instability they can readily extract electrons from other molecules in close proximity and cause cellular damage.

A single electron reduction of oxygen results in the formation of the superoxide radical. Normally, this molecule is kept at a low intracellular concentration through two mechanisms. The superoxide radical can either undergo spontaneous dismutation or in the presence of superoxide dismutase (SOD) it will catalytically breakdown into hydrogen peroxide (Klaassen, 2001). The superoxide radical can also react with nitric oxide to form reactive nitrogen species (RNOS) that can target proteins, lipids and DNA for damage (Klaassen, 2001; Thannickal, 2000).

Unlike the superoxide radical, hydrogen peroxide is much more thermodynamically stable thus being able to diffuse across biological membranes

(Thannickal, 2000). After its formation, hydrogen peroxide can be further reduced through one of three mechanisms. In the presence of the enzyme catalase, hydrogen peroxide can be catalyzed to form water and oxygen. It can also be detoxified by the enzyme glutathione peroxidase in the presence of glutathione to form water and oxidized glutathione. Lastly, if hydrogen peroxide is in the presence of either the superoxide radical or a transition metal such as copper or iron, it can be reduced to form the hydroxyl radical (Klaassen, 2001; Thannickal, 2000)

The hydroxyl radical is highly reactive having a half-life of about 10^{-9} seconds (Aruoma, 1998). Once produced it can and will react with any biological molecule it comes in contact with. As a result, it can cause detrimental effects on proteins, nucleic acids and polyunsaturated fatty acids (PUFAs) (Klaassen, 2001). Also, when it reacts with another molecule it generally creates another free radical causing more cellular damage (Aruoma, 1998).

1.3 Toxic Outcomes of Oxidative Stress

1.3.1 *Oxidative DNA Damage*

Normal cellular metabolism is a source of endogenous ROS which, may contribute to background levels of oxidative DNA damage detected in normal tissues. Phagocytic cells are also capable of generating ROS with the intention of targeting cells infected with bacteria or viruses; however, surrounding biomolecules may also be

detrimentally affected (Cooke *et al.*, 2007). The hydroxyl radical is highly reactive to DNA and it generates a variety of products from all four bases (Knaapen *et al.*, 2006). The hydroxyl radical reacts with DNA by addition to double bonds of DNA bases and abstraction of hydrogen atoms from the methyl group of thymine and each C-H bond of 2'-deoxyribose. Addition to the C5-C6 double bond of pyrimidines yields C5-OH and C6-OH adduct radicals and hydrogen abstraction from thymine yields an allyl radical. Pyrimidine radicals yield many product types that depend on redox properties, redox environment and reaction partners (Cooke *et al.*, 2007). These products are capable of causing mispairing with themselves and neighboring pyrimidines, generating several amino acid substitutions (Haghdoust, 2005; Mundt *et al.*, 2008).

The hydroxyl radical can add to the C4, C5 and C8 positions of purines to generate adduct radicals. A guanine radical cation can be formed from a C4-OH adduct radical of guanine that can react with 2'-deoxyribose in DNA by hydrogen abstraction to cause DNA strand breaks (Cooke *et al.*, 2007; Klaassen, 2001). Oxidation of the C8-OH adduct radicals competes with the unimolecular opening of the imidazole ring by scission of the C8-N9 bond, which is capable of inhibiting DNA replication (Cooke *et al.*, 2007; Klaassen, 2001; Tudek, 2003). Also the hydroxyl radical can abstract hydrogen atoms from the sugar moiety of DNA to cause super modifications and strand breaks (Klaassen, 2001). Oxidative DNA damage causes cellular changes in DNA conformation, repair mechanism activity, sequence context and incorporation of bases. A significant number of oxidative base lesions impact replication and transcription but can also cause mutagenic effects such as deletions, transversions, replicative blocks and loss of

heterozygosity (Cooke *et al.*, 2007). Oxidative base lesions are removed and repaired normally by base excision repair (BER) or nucleotide excision repair (NER), however the cell's capacity to repair DNA is limited (Cooke *et al.*, 2007).

1.3.2 Protein Oxidation

While oxidative damage to nucleic acids is subject to repair by efficient excision/insertion mechanisms, the repair of damaged proteins is limited to the reduction of oxidized derivatives of the sulfur-containing amino acid residues. Proteins have very specific conformations and patterns of folding that allow for their proper activity and function (Chevion, 2000). When ROS come in contact with proteins they oxidize their amino acids residues and form protein carbonyls. This modification can disrupt protein folding and change its structure thus resulting in a decrease or complete loss of enzymatic activity and/or function (Grune *et al.*, 1997). In most cases, these damaged proteins are targeted for degradation to amino acid constituents via endogenous proteases such as cathepsin c, calpain, trypsin and especially the 20s proteasome (Statdman & Levine, 2006).

Metal-catalyzed oxidation of proteins is a site-specific process that involves divalent cation binding sites on proteins occupied by redox-sensitive cations such as iron or copper. This is, in most cases, at or near the active site and renders the site susceptible to oxidation by ROS that will preferentially react with amino acid residues at the binding site, leaving the protein inactive (Statdman & Levine, 2006). Oxidation of proteins can

also occur as a consequence of oxidative cleavage of the peptide backbone via the α -amidation pathway, or cleavage associated with the oxidation of glutamyl residues. Secondary reactions with lipid peroxidation products can also oxidize proteins forming carbonyl derivatives. Oxidized proteins can further react with the α -amino group of lysine residues to form intra- or inter-molecular protein cross-linked derivatives. These derivatives are resistant to proteolytic degradation by the 20s proteasome and can inhibit proteosomal degradation of other oxidized proteins (Stadtman & Levine, 2006). The accumulation of modified proteins disrupts cellular function through a loss of catalytic function, structural integrity and/or the interruption of regulatory pathways (Stadtman & Levine, 2006).

1.3.3 Lipid Peroxidation

Lipid peroxidation is another hallmark of oxidative stress and is the result of free radicals oxidizing fatty acids. The formation of lipid peroxidation products leads to the spread of free radical reactions. The process of lipid peroxidation consists of three stages: initiation, propagation and termination (Catalá, 2006). The initiation phase of lipid peroxidation includes hydrogen atom abstraction. Lipids found in membranes, mainly phospholipids, contain polyunsaturated fatty acids (PUFAs), which are predominantly susceptible to peroxidation. The presence of a double bond in the fatty acid weakens the C-H bonds on the carbon atom nearby the double bond and thus facilitates a hydrogen atom subtraction. An initial reaction of the hydroxyl radical with a PUFA produces a lipid radical, which is converted into a lipid peroxy radical via oxygen fragmentation

(Catalá, 2006; Klaassen, 2001). The lipid peroxy radical can abstract hydrogen from a neighboring fatty acid to produce a lipid hydroperoxide and a second lipid radical. The lipid hydroperoxide formed can suffer a reductive cleavage by reduced metals such as iron, producing a lipid alkoxyl radical. Both alkoxyl and peroxy radicals stimulate the chain reaction of lipid peroxidation by abstraction of additional hydrogen atoms (Catalá, 2006; Halliwell *et al.*, 1993). Peroxidation of lipids can alter the assembly of the membrane, causing changes in permeability and fluidity, alterations in transport characteristics and inhibition of metabolic processes (Klaassen, 2001). Furthermore, lipid hydroperoxide can break down, frequently in the presence of reduced metals or ascorbate, to reactive aldehyde products such as 4-hydroxynoneal (HNE) and malondialdehyde (MDA). Lipid peroxidation is one of the major outcomes of free radical-mediated injury to tissue and can greatly alter the physicochemical properties of membrane lipid bilayers, resulting in severe cellular dysfunction (Catalá, 2006; Klaassen, 2001)

1.3.4 Thiol Depletion

A cell's response to stress involves changes in thiol content due to the susceptibility of cysteine residues to oxidation. Glutathione (GSH) is the most abundant non-protein thiol in most cells and has a redox potential of -0.23 Volts (Bilska *et al.*, 2005). GSH is important in antioxidant defense, xenobiotic and eicosanoid metabolism and regulation of cell cycle and gene expression (Dickinson *et al.*, 2002). It can form conjugates with a variety of electrophilic compounds non-enzymatically, when the electrophile is very reactive or more commonly through the action of glutathione S-

transferases (GST) (Jones, 2008). Several proteins can be regulated by the introduction or removal of GSH; under oxidative stress many proteins undergo S-glutathionylation catalyzed by glutaredoxins. GSH can also be used as a reductant for selenium-dependent GSH peroxidases (GPx) and some GST reactions that produce glutathione disulfide (GSSG) (Jones, 2008). GSSG is reduced back to GSH by accepting an electron from NADPH catalyzed by glutathione reductase. This reaction allows for the restoration of GSH, however, depletion can occur under oxidative stress through conjugation or excretion of GSSG and GSH conjugates through an ATP-dependent transport mechanism (Dickinson *et al.*, 2002).

1.4 Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a small, neutral molecule permeable to cell membranes with both toxicological and physiological signaling roles. It is generated directly in cells by oxidoreductases, dismutation of the superoxide radical, leakage from mitochondrial electron transport chain and redox cycling of quinones and several flavoproteins (Forman, 2007). Once formed, H_2O_2 can be easily converted to hydroxyl radical that can react with DNA, proteins or lipids or cause the depletion of intracellular thiol content leading to cellular damage and/or cell death. A number of studies have shown that H_2O_2 activates a number of signaling cascades by stimulating the formation of the SHC-Grb2-Sos complex that activates Ras (Chetsawang *et al.*, 2010; Janssen *et al.*, 1997). It also has been shown to activate the nuclear factor-kappa beta (NF- κ B) signaling pathway by inducing tyrosine phosphorylation of the inhibitory subunit (I κ B) and serine

phosphorylation of the p65 subunit of NF- κ B. NF- κ B can then be translocated into the nucleus where it activates a large constituency of target genes involved in inflammation, angiogenesis and apoptotic functions (Allen & Tresini, 2000; Chetsawang *et al.*, 2010; Takada *et al.*, 2003; Weinberg, 2007). Protein tyrosine phosphatases (PTP) are important regulators of signal transduction under normal and pathophysiological conditions (Forman, 2007). PTPs have a highly conserved region in the catalytic domain where either an oxidation or reduction of the cysteine residue renders the molecule inactive. H₂O₂ stimulated cells undergo glutathionylation of the cysteine residue, which prevents the modification of the active site resulting in an increase of protein phosphorylation (Allen & Tresini 2000; Forman, 2007).

1.5 Paraquat

In 1882 paraquat (1,1'-dimethyl-4,4'-bipyridylium dichloride; PQ) was first synthesized by Weidel and Rosso. Michaelis and Hill then discovered its redox properties in 1933. But it wasn't until 1955 at the Jealott's Hill International Research Center in Bracknell, UK, that PQs herbicidal properties were uncovered (Dinnis-Oliveira *et al.*, 2008). In 1962 PQ was made available in the market through the Plant Protection Division Ltd of Imperial Chemical Industries (ICI; now Syngenta) under the trade name Gramoxone. There are currently about 100 countries that PQ is registered and sold to, including the USA, Canada, and Australia (Paraquat Information Center).

PQ belongs to the chemical family bipyridylium and consists of 2 quaternized pyridine rings. It is a non-selective contact herbicide that destroys cellular membranes of green plants by interfering with the intracellular electron-transfer system (Klaassen, 2001). PQ inhibits the reduction of NADP to NADPH during photosynthesis, resulting in the formation of ROS. The ROS can react with the unsaturated lipids in the cellular membrane leading to its destruction and cell death (Suntres, 2002; Autor, 1977).

Cellular toxicity of PQ is due to its cyclic reduction-oxidation; PQ is reduced enzymatically mainly by NADPH-cytochrome P₄₅₀ reductase and xanthine oxidase. This reaction forms a PQ cation free radical that is rapidly re-oxidized in the presence of oxygen to form the superoxide radical and subsequently hydrogen peroxide that leads to the generation of the hydroxyl radical (Hoet & Nemery, 2000). These events lead to the oxidation of NADPH and thiol groups and damage to lipids, proteins and DNA (Suntres, 2002; Klaassen, 2001).

PQ toxicity is selective to pulmonary tissues due to a unique polyamine uptake system (PUS) found on alveolar epithelial type I and type II cells. Polyamines are known to regulate cell growth, division and differentiation and are thought to play a role in the differentiation of type II to type I pneumocytes. To act as a substrate for the PUS, a molecule must have the following specific characteristics for binding and consequently, transport: (1) two or more positively charged nitrogen atoms, (2) maximum positive charge is around the nitrogen atoms, (3) there is a non-polar group between charges and (4) minimal steric hindrance. PQ has these characteristics although the optimum distance

between nitrogen centers is 4 methylene groups (~0.622nm) and PQ has seven (~0.702nm). This results in a lower affinity for the uptake system than polyamines, but PQ is still recognized as a substrate and accumulated into the lung through this transport pathway (Suntres, 2002; Hoet & Nemery, 2000).

There are two distinct phases in the development of PQ-induced pulmonary lesions, which are the destructive and proliferative phases. The first pulmonary changes caused by PQ are observed in type I alveolar epithelial cells. These cells exhibit swelling accompanied by an increase in their content of mitochondria and ribosomes, suggesting an increase in metabolic activity. Initially mitochondria swell, followed by cellular degeneration and cytoplasmic edema causing the rupture of type I cells and exposure of the basement membrane (Autor, 1977; Suntres, 2002). PQ compromises lung function early on with the destruction of type I cells that form the alveolar vesicles for gas exchange (Klaassen, 2001). Damage to type II cells lags behind type I cell injury, but begins with mitochondrial swelling and loss of the contents of the lamellar bodies before cell death (Suntres, 2002). The destruction of type II cells results in the decrease of surfactant secretion, transport of water and ions and epithelial regeneration (Klaassen, 2001).

The inflammatory response that arises during the destructive phase, and is maintained into the proliferative phase, involves rapid and extensive influx of inflammatory cells mainly leukocytes, macrophages and eosinophils into the interstitium and alveolar spaces. The proliferative phase involves the development of extensive

fibrosis as a compensatory repair mechanism to the damaged alveolar epithelial cells. Profibroblasts first appear in the alveolar sacs where they proliferate and differentiate into mature fibroblasts to lay down collagen and ground substance to produce fibrosis. This causes a loss of pulmonary elasticity, respiratory impairment and inefficient gas exchange leading to anoxia (Suntres, 2002).

PQ is also known to inhibit the transfer of electrons along complex I of the electron transport chain causing the inhibition of ATP synthase. Suppression of ATP synthase compromises ATP-dependent pumps that cause the loss of ionic and volume-regulatory controls (Klaassen, 2001). Depletion of cellular ATP reserves denies endoplasmic and plasma membrane Ca^{2+} pumps of fuel, causing the elevation of Ca^{2+} in the cytoplasm. High cytosolic Ca^{2+} levels stimulate Ca^{2+} uptake into the matrix via the Ca^{2+} uniporter, causing the mitochondrial membrane potential to decline, and further hindering ATP synthase (Schweizer & Richter, 1996). Elevated Ca^{2+} levels can also activate hydrolytic enzymes such as proteases and phospholipases that degrade proteins and phospholipids. It has also been shown that Ca^{2+} can lock topoisomerase II in a form that cleaves DNA (Klaassen, 2001). PQ is known to undergo redox cycling that directly generates ROS, however it is also capable of generating ROS through elevated Ca^{2+} levels. Ca^{2+} activates dehydrogenases in the citric acid cycle, accelerating hydrogen output from the citric acid cycle and therefore electrons into the electron transport chain (Giacomello *et al.*, 2007). This, in addition to the suppression of ATP synthase activity causes the increased formation of the superoxide radical by the electron transport chain.

1.6 Treatment of Paraquat Toxicity

Currently there is no antidote used for the treatment of PQ poisoning in humans. Most antidotes aim for detoxification of the superoxide radical, subsequent ROS and/or modification of PQ toxicokinetics (Suntres, 2002). Treatments such as induced emesis, gastric lavage, administration of mineral absorbents, hemodialysis and hemoperfusion has been found to be unsuccessful or disappointing (Bismuth *et al.*, 1982; Honore *et al.*, 1987; Klaassen, 2001; Suntres, 2002).

1.6.1 Superoxide Dismutase and Mimetic Enzymes

The superoxide radical produced by PQ or other sources is normally kept under control by SOD enzymes. The treatment of PQ-induced injury with SOD has given variable results. It is thought that its high molecular mass and ionic charge prevent intracellular transport and adherence to targets. To avoid these problems, low-molecular weight metalloporphyrin SOD mimetics have been used to treat oxidative stress-induced injuries. Specifically, Day and Crapo (1996) demonstrated that a SOD mimetic had potential in protecting against PQ-induced lung injury. The mimetic was found to penetrate cell membranes, retain activity within the cell and also protect endothelial cells.

1.6.2 Antioxidant Vitamins

1.6.2.1 Ascorbic Acid (Vitamin C)

Ascorbic acid acts as a two-electron reducing agent, capable of scavenging free radicals and regenerating other small molecule antioxidants. High concentrations of ascorbic acid have been found in the fluid of the lungs that help protect against air-borne toxic chemicals (Carr & Frei 1999; Suntres 2002). A short pretreatment with ascorbic acid has demonstrated a protective effect on tissue damage through a reduction in lipid peroxidation by-products (Matkovacs *et al.*, 1980). However, the treatment of PQ-induced toxicity has shown an accelerated generation of radicals, exacerbating the oxidative damage. This acceleration has been attributed to the redox cycling of free transition metal ions in the aqueous phase. Pretreatment with an iron-chelating agent such as, desferoxamine has been shown to reduce the interaction between the free transition metals and ascorbic acid and as a result a significant reduction in PQ-toxicity (Kang *et al.*, 1998; Suntres, 2002).

1.6.2.2 α -Tocopherol (Vitamin E)

α -Tocopherol is a lipid-soluble vitamin that exercises its antioxidant effects by scavenging free radicals and stabilizing membranes containing PUFAs. Multiple studies have shown that animals with a α -tocopherol deficiency have an increased likelihood of

developing acute PQ toxicity. The potentiation of acute PQ toxicity by α -tocopherol deficiency was reversed by its administration (Block, 1979; Suntres, 2002).

Under normal conditions, further supplementation of α -tocopherol in animals has shown to be ineffective in protecting against lipid peroxidation or acute mortality in PQ-induced toxicity. This ineffectiveness could be related to its solubility; lipid-soluble antioxidants take longer to diffuse through cellular membranes (Suntres, 2002). Further studies with water-soluble analogues of α -tocopherol and/or liposomal preparations might lead to better treatments (Newmark *et al.*, 1975; Suntres *et al.*, 1992).

1.6.3 Low Molecular Weight Thiol-Containing Antioxidants

1.6.3.1 *Glutathione*

Compounds containing thiol groups are among the most important endogenous antioxidants. GSH is the most abundant non-protein thiol and it provides intracellular protection against ROS and other free radicals. (Anderson, 1997; Mulier, 1998; Suntres, 2002). The modulation of cellular GSH/GSSG levels is capable of inducing several enzymes/proteins involved in the redox system of the cell and their genes (Rahman, 2006). GSH can function as a nucleophile to form conjugates with xenobiotics and/or its metabolites as well as functioning as a reductant in the metabolism of hydrogen peroxide, catalyzed by glutathione peroxidase (Klaassen, 2001; Suntres 2002; Suntres, 2006).

Studies done *in vitro* with alveolar type II cells have shown that supplementation with exogenous GSH protects against PQ-induced injury (Hagen *et al.*, 1986). However, actual treatment of PQ or other oxidant injury with exogenous GSH is restricted by its rapid hydrolysis and inability to cross cell membranes (Smith *et al.*, 1992). Supplementation of animals with GSH precursors over prolonged periods of treatment has resulted in an increase of the intracellular GSH pool and a decrease in the susceptibility to oxidant-induced injury (Anderson & Luo, 1998).

1.6.3.2 *N*-Acetylcysteine

N-Acetylcysteine (NAC) is an acetylated variant of the amino acid L-cysteine and is a cell-permeable precursor of GSH (Mitsopoulos & Suntres, 2011). Metabolites of NAC stimulate GSH synthesis, promote detoxification and directly act as free radical scavengers. In pretreated animals, NAC has shown protective effects against PQ toxicity by decreasing the amount of edema and cellular infiltration in the lung, compared to without pretreatment (Wegener *et al.*, 1988). In another study the administration of NAC to animals treated with PQ did not affect the survival rate, but did delay the release of chemoattractants and decreased the infiltration of inflammatory cells (Hoffer *et al.*, 1993, 1996; Yeh *et al.*, 2006).

1.7 α -Lipoic Acid

1.7.1 *Background*

Purified in 1951 by Reed *et al.*, α -lipoic acid (thiotic acid; LA) is an eight-carbon di-thiol that is both lipophilic and hydrophilic (Bilska *et al.*, 2005). LA is an essential co-factor in α -ketoacid dehydrogenase complexes and the glycine cleavage system, where it is covalently attached in an amide linkage to a lysine residue (Packer *et al.*, 1995,1998; Moini *et al.*, 2002). α -Lipoic acid is currently being used in pre-clinical and clinical trials for therapy of diabetes and diabetic neuropathy (Bilska *et al.*, 2005). Also, this compound has become of interest in sports medicine, as well as in the beauty-care industry (Bilska *et al.*, 2005). The most abundant plant sources of LA are spinach, followed by broccoli and tomatoes (Moini *et al.*, 2002). The highest concentration of LA in animal tissues was found in the kidney, heart and liver (Packer *et al.*, 1995).

The low redox potential of the LA/DHLA system is conclusive evidence of strong antioxidant properties. It has high reactivity towards free radicals, the capability of regenerating ascorbic acid and α -tocopherol and furthermore it can elevate intracellular GSH content (Bilska *et al.*, 2005). LA structural characteristics enable it to be easily absorbed in the stomach and cross the blood-brain barrier without showing toxic effects at doses used for prophylactic and therapeutic purposes. Also, many experimental and clinical studies have demonstrated beneficial effects of LA in diseases such as diabetes,

atherosclerosis, heart disease, cataract, neurodegenerative diseases, liver disease and AIDS (Bilska *et al.*, 2005; Packer *et al.*, 1998).

1.7.2 Chemistry, Uptake and Metabolism

LA is a disulfide derivative of octanoic acid and it forms an intramolecular disulfide bond in its oxidized form (Bilska *et al.*, 2005; Moini *et al.*, 2002). There is high electron density associated with the sulfur atoms that confers high tendency to reduce other redox-sensitive molecules (Packer *et al.*, 1995).

Orally administered LA is absorbed and transported to tissues, where it is taken up by the cells. Exogenous LA enters the mitochondrial matrix where dihydrolipoamide dehydrogenase reduces LA using electrons from NADH to form dihydrolipoic acid (DHLA) (Moini *et al.*, 2002). Once LA is reduced, DHLA is released into the extracellular fluids (Packer *et al.*, 1995; Moini *et al.*, 2002). Unlike other antioxidants such as ascorbic acid, quenching free radicals does not destroy DHLA, rather it is recycled from LA (Moini *et al.*, 2002). The reduction of LA has also been observed in human erythrocytes, which lack mitochondria (Packer *et al.*, 1998; Moini *et al.*, 2002). In the erythrocyte system glutathione reductase, which exists primarily in the cytosol, was found to catalyze a slower reduction of LA using NADPH as the electron donor (Packer *et al.*, 1998). Thus, the mechanism of reduction of exogenous LA is unique; in different cellular compartments, LA is reduced by different enzymatic systems.

LA is metabolized into shorter chain homologues that are β -oxidation products such as bisnorlipoate, tetranorlipoate and β -hydroxybisnorlipoic (Packer *et al.*, 1995, 1998; Moini *et al.*, 2002). It is unknown how much LA is converted into these metabolites, but they do have the potential to scavenge free radicals or exhibit pro-oxidant effects in the absence of an iron chelator (Packer *et al.*, 1998).

1.7.3 Antioxidant Activities of Lipoic Acid

1.7.3.1 Direct Radical Scavenging and Metal Chelation

LA scavenges hydroxyl radicals, hypochlorous acid (HOCl), singlet oxygen and nitric oxide, while DHLA is capable of scavenging hypochlorous acid, peroxy, superoxide, hydroxyl and nitric oxide radicals. Both are also capable of chelating a number of transition metals.

In an experiment by Suzuki *et al.* (1991), the hydroxyl radical was generated by the reaction of hydrogen peroxide and iron and detected by electron spin resonance (ESR) using the spin-trapping agent 5,5-Dimethylpyrroline-N-oxide (DMPO). Both LA and DHLA (0.2mM), completely eliminated the DMPO-OH adduct signal demonstrating their ability to scavenge the hydroxyl radical. Several other studies have looked at the modulation of HOCl- and peroxynitrite- induced inactivation of α 1-antiproteinase by LA and DHLA. At concentrations of 0.01-0.5mM, LA and DHLA efficiently protected

against the inactivation of α 1-antiproteinase, proving their ability to scavenge both HOCl and RNOS (Nakagawa *et al.*, 1998; Scott *et al.*, 1994; Whiteman *et al.*, 1996).

The superoxide radical was generated using xanthine-xanthine oxidase and detected by ESR using DMPO. Total elimination of the DMPO-OOH adduct signal was observed for DHLA but not LA (Suzuki *et al.*, 1991). However, only LA was proven to scavenge singlet oxygen by generating rubrene autooxidation and photosensitized oxidation of methylene (Stevens *et al.*, 1974; Stary *et al.*, 1975).

Lastly, using two different azoinitiators, Kagan *et al.* (1992) were able to determine the effects of LA and DHLA on peroxy radicals in both, aqueous and lipid environments. Unlike LA, DHLA was able to efficiently and directly scavenge the peroxy radicals generated in both these environments.

As demonstrated by Ou *et al.* (1994), LA can decrease copper-induced oxidation of ascorbic acid and copper-induced lipid peroxidation. LA is also capable of decreasing site-specific iron-induced degradation of deoxyribose, indicating that LA is capable of chelating copper and iron (Scott *et al.*, 1994). In the presence of the iron chelator desferoxamine, DHLA was able to drastically decrease the amount of lipid peroxidation in microsomes. In the absence of the iron chelator, DHLA had no effect on lipid peroxidation suggesting it chelates iron (Suzuki *et al.*, 1993; Bonomi *et al.*, 1986). In a similar experiment using isolated rat hepatocytes, DHLA was able to ameliorate cadmium-induced lipid peroxidation and increase cellular GSH (Muller & Menzel, 1990).

1.7.3.2 Interactions With Other Antioxidants

With a redox potential of -0.29V, the DHLA/LA redox couple is a potent reductant, even more so than the GSH/GSSG couple, which has a redox potential of -0.23V (Bilska *et al.*, 2005). This means that DHLA is capable of reducing GSSG to GSH, but GSH is incapable of reducing LA to DHLA. Not only is it capable of regenerating GSH, but also it can regenerate other antioxidants such as ascorbic acid and α -tocopherol. α -Tocopherol is important in protecting cellular membranes from lipid peroxidation. DHLA has been shown to weakly reduce tocopheroxyl radicals at the membrane-water interface. However, the major recycling of α -tocopherol by DHLA in biological systems occurs indirectly through the reduction of glutathione, vitamin C, ubiquinol, NADPH or NADH (Bilska *et al.*, 2005).

1.7.3.3 Accelerated Synthesis of Glutathione

Maintenance of cellular GSH depends on *de novo* synthesis that is limited by the intracellular concentration of cysteine (Han *et al.*, 1997). Under oxidative stress, cysteine is oxidized to cystine and exported from cells by an amino acid transporter. Cellular exposure to LA results in the uptake and metabolic reduction of LA to DHLA by NADH and NADPH. DHLA is released into the extracellular space where it subsequently reduces cystine to cysteine. Cysteine can be taken up by the amino acid transporters and incorporated into GSH synthesis (Han *et al.*, 1997; Packer *et al.*, 1998)

1.7.3.4 Modulating Transcription Factor Activity

LA and DHLA have the ability to modulate transcription factor activity. NF- κ B is regulated through redox mechanisms and its activation can be influenced by thiol content. For NF- κ B to become activated, it must become phosphorylated and disassociate from an inhibitory subunit, I- κ B (Sen *et al.*, 1996; Packer *et al.*, 1998). In a study by Sen *et al.* (1996), hydrogen peroxide was shown to increase the intracellular calcium levels that can result in the phosphorylation of NF- κ B, and degradation of I- κ B. Pretreatment with LA decreased the oxidant-induced influx of calcium and therefore suppressed NF- κ B activation. Binding of the NF- κ B transcription factor to DNA involves cysteine residues that can be regulated through oxidation and reduction reactions. DHLA has been shown to reduce cystine to cysteine, which can stimulate the binding of NF- κ B to DNA (Packer *et al.*, 1995). However, incubation of cells with LA induced phosphorylation of Akt that was accompanied by the inhibition of LPS-induced NF- κ B DNA binding activity and up-regulation of TNF- α and monocytes chemoattractant protein 1.

Objectives

Objective I: Characterize hydrogen peroxide or PQ toxicity as a mechanism of oxidative stress in A549 lung epithelial cells. Cell viability will be assessed by the MTT assay analysis of a concentration- and time-course of H₂O₂/PQ exposure. Further characterization of H₂O₂/PQ toxicity will be carried out by assessing DNA fragmentation, mitochondrial membrane potential, apoptosis, cell cycle and lipid peroxidation.

Objective II: Examine the effectiveness of lipoic acid (LA) in protecting against oxidative stress. The effect of LA on cell viability will be assessed using the MTT assay to determine the concentration range in which LA has no detrimental effect on cell viability. H₂O₂/PQ-challenged A549 cells co-treated with LA will be assessed by measuring cell viability via the MTT assay. The mechanism(s) of LA cytoprotection against H₂O₂/PQ will be investigated by assessing DNA fragmentation, mitochondrial membrane potential, apoptosis, cell cycle and lipid peroxidation. The uptake and cellular distribution of LA will be assessed by chemical analytical procedures.

Methods

1.1 Cell culture

Human alveolar type II-like epithelial A549 cells (American Type Culture Collection # CCL-185; ampule passage no. 80; ATCC, Manassas, VA, USA) were maintained in Costar 0.2 μ M vent cap cell culture flasks (Corning, NY, USA) with standard Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 10% iron-fortified bovine calf serum (SAFC Biosciences, Lenexa, KS, USA), 2mM L-glutamine (Gibco, Carlsbad, CA) and antibiotic/antimycotic (100 U/mL penicillin, 100 μ g/mL streptomycin and 0.25 μ g/mL amphotericin B; Gibco). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air until 80% confluence. Cell counts and viabilities were assessed using a Vi-Cell XR Cell viability Analyzer (Beckman Coulter, Mississauga, ON, Canada).

1.2 Hydrogen Peroxide Preparation

The stock solution of 8.82M hydrogen peroxide (Fisher Scientific) was serially diluted in media (serum-free) to formulate specific treatment concentrations.

1.3 Paraquat (PQ) preparation

PQ (Paraquat dichloride, Sigma-Aldrich) was dissolved in ddH₂O to produce a 100 mM stock solution that was stored at 4°C in the absence of light. The stock solution of PQ was added to serum-free culture media to formulate treatment-specific concentrations.

1.4 α -Lipoic Acid (LA) preparation

LA ((\pm)- α -Lipoic Acid, Sigma-Aldrich) was dissolved in 70% ethanol to produce a 145mM solution that was further diluted with 1X phosphate buffered saline (Fisher Scientific) to 18.12mM stock solution and the pH was adjusted to 7.2 using SevenEasy pH (Mettler Toledo, Switzerland). A new stock solution was prepared for each experiment and was added to serum-free culture media to formulate specific treatment concentrations.

1.5 Cell Viability

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow tetrazolium salt that is taken up by cells due to its net positive charge and the plasma membrane potential. It is then reduced in metabolically active cells to form insoluble purple formazan crystals by intracellular NAD(P)H-oxidoreductases. The colour produced can be quantified by spectrophotometric means (Berridge *et al* 1993, 2005).

A549 cells were seeded into sterile flat-bottom 96-well plates (Corning) at a concentration of 10,000 cells/well and incubated overnight. To determine the effect of LA, H₂O₂ and/or PQ on cell viability, cells were treated with control or LA-, H₂O₂- or PQ-containing media (serum-free). To determine the effect of LA co-treatment on viability of H₂O₂- or PQ-treated cells, cells were treated with control or LA-containing media incubated for 5 minutes and H₂O₂- or PQ-containing media (various concentrations and times; serum free) for 6 hours. Following treatment, a 5mg/mL stock solution of MTT (Thiazolyl Blue Tetrazolium Bromide; Sigma-Aldrich, St. Louis, MO USA) was made and 10% of the final volume in each well was added and incubated at 37°C for an additional 4 hours. During this time the MTT was converted to purple formazan crystals in viable cells. After the incubation period, the media was aspirated and 50 µL/well of DMSO was added to each well to solubilize the formazan crystals. Cells were then agitated for 10 minutes, following which absorbance was measured spectrophotometrically at a wavelength of 570nm using a PowerWave XS Spectrophotometer (BioTek, Winooski, VT, USA). Cell viability was assessed relative to control wells, which were taken to have 100% viability.

1.6 High Performance Liquid Chromatography (HPLC)

Liquid chromatography was performed on a Varian Pro Star High-Performance Liquid Chromatograph (HPLC) composed of UV mono-wavelength detector (Nashville, TN, USA). Conditions for the optimization of LA detection using HPLC were based on HPLC methodology outlined by Aboul-Enein & Hoenen (2004). Briefly, a stock solution

of LA (18.12 mM) was diluted in serum-free media to specific concentrations then filtered by a Millipore membrane filter (0.22 μ M). Samples were injected onto a C18 reverse phase column (4.6 mm D. \times 15.0 cm length) at 25°C. The mobile phase consisted of acetonitrile: potassium monophosphate [0.05 M] pH 2.5 (45: 55 v/v) at a flow rate of 1.0 mL/min. The concentration of LA was measured at 332 nm.

1.7 Mitochondrial membrane potential

An early indicator of apoptosis is the dissipation of the mitochondrial membrane potential, which is a driving force for ATP synthase (Giacomello *et al* 2007; Schweizer & Richter 1996). JC-1 (5,5',6,6'-tetrachloro-1,1',3',3'-tetraethylbenzimidazolyl carbocyanine iodine), is a cationic dye capable of entering mitochondria and reversibly changing fluorescence from red to green as membrane potential increases. Viable cells with negative mitochondrial membrane potentials form J-aggregates with the JC-1 dye, causing an increase in red fluorescence. Cells undergoing membrane depolarization results in the dissolution of these J-aggregates causing a decrease in red fluorescence.

Using the JC-1 Mitochondrial Membrane Potential Assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). Cells were seeded into sterile flat-bottom 6-well plates at 0.5×10^6 cells/well and incubated overnight. Cells were then treated with control, LA- (0.1mM, 0.5mM or 1mM) and/or PQ-containing (0.5mM, 1mM, 2mM or 5mM) serum-free media for 6 hours. Following treatment cells were stained for 30 minutes with JC-1 dye. Cells were harvested via trypsinization and centrifuged at $400 \times g$ for 5 minutes at

room temperature followed by two washes with assay buffer (Cayman Chemical). Cell pellet was then resuspended in 500 μ L assay buffer for flow cytometric analysis using the FL1-H and FL2-H channels of a BD FACSCalibur Flow Cytometer (BD Biosciences) with BD CellQuest Pro Software. A minimum of 10,000 events was acquired per trial. Mitochondrial depolarization was indicated by a decrease in red fluorescence intensity due to a decrease in the formation of red fluorescent J-aggregates.

1.8 Lipid Peroxidation

Lipid peroxidation (LPO) is a well-established mechanism of cellular injury and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides are unstable and decompose to form a series of reactive carbonyl compounds. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) and 4-hydroxyalkenols (HAE) upon decomposition. LPO will be assessed using chromogenic reagent, N-methyl-2-phenylindole (R1) from BIOXYTECH LPO-586 Colorimetric Assay Kit (OxisResearch, Burlingame, CA, USA). One molecule of MDA or HAE reacts with 2 molecules of R1 at 45°C to yield a stable chromophore that can be detected spectrophotometrically at 585nm.

Cells were seeded into sterile flat-bottom 6-well plates (Corning) at 10^6 cells/well and incubated overnight. Following incubation cells were treated with control, LA-containing (0.1 mM, 0.5 mM or 1 mM) and/or PQ-containing (5 mM) or co-treated with LA and PQ in serum-free media for 6 hours. Following treatment cells were detached

from the plate surface using Fisherbrand disposable sterile cell scrapers (Fisher Scientific) and suspended in PBS. To prevent sample oxidation 10 μ L of 0.5 M butylated hydroxytoluene (BHT, LKT Laboratories Inc, St. Paul, MN, USA) was added per mL of cell homogenate. Cells were then lysed via sonication (20s, 100% amplitude; Sonic Dismembrator Model 500, Fisher Scientific, Pittsburgh, PA, USA) and centrifuged at $3000 \times g$ at 4°C for 10 minutes to collect the cell homogenate. The supernatant was collected and stored at -80°C overnight. Frozen samples were thawed and divided into 200 μ L aliquots. Once divided into aliquots, R1 and R2 reagents were added to samples and incubated at 45°C for 60 minutes. Following the incubation, samples were centrifuged at $15,000 \times g$ for 10 minutes at room temperature and supernatant was collected. Samples were analyzed immediately by spectrophotometry at 586 nm using a Genesys 10 UV spectrophotometer (Thermo Scientific, Madison, WI, USA). LPO was indicated by an increase in net absorbance at 586 nm.

1.9 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL)

A biochemical hallmark of apoptosis is the activation of nucleases that eventually degrade the nuclear DNA into fragments of approximately 200 base pairs in length. DNA breaks expose a large number of 3'-hydroxyl ends that can then serve as a starting point for terminal deoxynucleotidyl transferase (TdT) to add deoxyribonucleotides in a template-independent fashion. Addition of a deoxythymidine analog 5-bromo-2'-deoxyuridine 5'-tri-phosphate (BrdUTP) to the TdT reaction serves to label the break

sites. Once BrdU is incorporated into the DNA an anti-BrdU antibody can detect it using standard immunohistochemical techniques.

DNA fragmentation was assessed using the APO-BrdUTM TUNEL Assay Kit (Molecular Probes, Inc, Eugene, OR, USA). Cells were seeded into sterile flat-bottom 6-well plates (Corning) at 10^6 cells/well and incubated overnight. Cells were treated with control, LA-containing (0.1 mM, 0.5 mM or 1 mM) and/or PQ-containing (0.5 mM, 1 mM, 2 mM, or 5 mM) serum-free media for 6 hours. Cells were suspended in 0.5 mL of PBS and 5 mL of 1% paraformaldehyde in PBS was added and placed on ice for 15 minutes. Following the incubation, cells were washed twice in PBS and resuspended in 0.5 mL PBS. To this suspension, 5 mL of ice-cold 70% ethanol was added and stored overnight in -80°C. The following day, the ethanol was removed with two washes of 1 mL wash buffer. Cells were resuspended in 50 μ L of the DNA-labeling solution and incubated for 60 minutes at 37°C. Samples were shaken every 15 minutes to keep the cells in suspension. At the end of the incubation period, cells were rinsed twice with 1 mL of the rinse buffer and resuspended in 100 μ L of the antibody staining solution. Cells were incubated for 30 minutes in dark at room temperature. The cell suspension was then diluted with 400 μ L of rinse buffer and analyzed immediately by flow cytometry on a BD FACSCalibur Flow Cytometer (BD Biosciences) using BD CellQuest Pro Software on the FL1-H channel. DNA fragmentation was indicated by an increase in green fluorescence due to the incorporation of AlexaFluor*488 dye-labeled anti-BrdU antibody into DNA break sites.

1.10 DNA Cell Cycle

Cell cycle distribution measurements can be combined to provide an alternative DNA fragmentation analysis. Propidium iodide (PI) binds DNA and provides a rapid and accurate means for quantitating both total nuclear DNA content and the fraction of cells in each phase of the cell cycle. PI is unable to penetrate an intact membrane so ethanol, an agglutinating fixative, permeabilizes the cell membrane so PI can enter the cell and intercalate into DNA. These cells with DNA fragments can then be observed as a hypodiploid or 'sub-G1' peak in a DNA histogram.

Cells were seeded into sterile flat-bottom 6-well plates (Corning) at 10^6 cells/well and incubated overnight. Cells were treated with control, LA-containing (0.1 mM, 0.5 mM or 1 mM) and/or PQ-containing (0.5 mM, 1 mM, 2 mM, or 5 mM) serum-free media for 6 hours. Following treatment cells were harvested via trypsinization, washed once with PBS and centrifuged for 5 minutes at 1500 rpm. The cell pellet was resuspended in 500 μ L of cold PBS and 4.5 mL of cold 70% ethanol was added dropwise while vortexing. Samples were then left at 4°C overnight following which, they were washed twice in PBS and incubated in 500 μ L of PI staining solution at room temperature for 30 minutes in the dark. Following the incubation samples were analyzed by flow cytometry on a BD FACSCalibur Flow Cytometer (BD Biosciences) using BD CellQuest Pro Software on the FL2-A channel. A minimum of 10,000 events per trial was acquired. Cell Cycle histograms that displayed a sub-G1 peak were indicative of DNA fragmentation.

1.11 Annexin V FITC

Changes to the plasma membrane occur in the early stages of apoptosis, one of which is the translocation of phosphatidylserine (PS) from the inner to outer leaflet of the cytoplasmic membrane. Annexin V is a Ca^{2+} -dependent phospholipid binding protein that has a high affinity for PS. In apoptosis the plasma membrane stays intact, while in later stages of cell death cellular membrane integrity is lost and becomes leaky resulting from either apoptotic or necrotic processes. Staining with Annexin V-FITC is typically used in conjunction with a live/dead dye such as propidium iodide that is membrane impermeant. Viable cells with intact membranes exclude PI, whereas membranes of dead and damaged cells are permeable to PI allowing for the distinction between early apoptotic and necrotic cells.

Cells were seeded into sterile flat-bottom 6-well plates (Corning) at 10^6 cells/well and incubated overnight. Cells were treated with control, LA-containing (0.1 mM, 0.5 mM or 1 mM) and/or PQ-containing (0.5 mM, 1 mM, 2 mM, or 5 mM) serum-free media for 6 hours. Following treatment cells were harvested via trypsinization, washed once with cold PBS and incubated in 100 μL of Annexin V incubation reagent for 15 minutes in the dark at room temperature. Following incubation, samples were diluted with 400 μL of 1X binding buffer and immediately analyzed by flow cytometry on a BD FACSCalibur Flow Cytometer (BD Biosciences) using the BD CellQuest Pro Software on the FL1-H (FITC) and FL2-H (PI) channels. A minimum of 10,000 events per trial

was acquired. Cells that are viable are FITC⁻/PI⁻ while cells in early apoptosis are FITC⁺/PI⁻ and cells in late apoptosis or undergoing necrosis are FITC⁺/PI⁺.

1.12 Intracellular Calcium

Toxicants are capable of elevating the cytoplasmic Ca²⁺ levels by promoting Ca²⁺ influx into or inhibiting Ca²⁺ efflux from the cytoplasm. Continuous elevation of intracellular Ca²⁺ levels is damaging because it can cause the depletion of energy reserves, dysfunction of microfilaments, activation of hydrolytic enzymes and/or generation of ROS and RNS. Fluo-3AM is a cell permeable dye that enters the cell where the lipophilic blocking group is cleaved by nonspecific esterases to release the Ca²⁺ sensitive form of the dye. Fluo-3 will exhibit a large fluorescence intensity increase upon Ca²⁺ binding determined using green fluorescence detectors.

Cells were seeded into sterile flat-bottom 6-well plates (Corning) at 10⁶ cells/well and incubated overnight. Following incubation cells were treated with control, LA-containing (0.1 mM, 0.5 mM or 1.0 mM) and/or PQ-containing (1.0 mM, 2.0 mM, or 5.0 mM) in serum-free media for 6 hours. Following treatment cells harvested via trypsinization and centrifuged for 7 minutes at 400 g. Cells were then resuspended at 10⁶ cells/200 µL in media. Cells were then loaded with 2 µM Fluo-3AM at 37°C for 45 minutes, with gentle mixing every 15 minutes. After incubation, an additional 200 µL of room temperature medium was added then samples were analyzed immediately by flow cytometry on a BD FACSCalibur Flow Cytometer (BD Biosciences) using BD CellQuest

Pro Software on the FL1-H channel. A minimum of 10,000 events was acquired per trial. An increase of intracellular Ca^{2+} levels was indicated by an increase in green fluorescence intensity.

1.13 Gas Chromatography – Mass Spectrometry (GC-MS)

Cells were seeded into sterile T-75 flasks (Corning) at 3×10^6 cells and incubated overnight. Following incubation, cells were treated with 0.5 mM LA in serum-free media for 6 hours. Following treatment cells were harvested via trypsinization and centrifuged for 5 minutes at $500 \times g$. Cells were washed with 2 mL PBS and centrifuged for 5 minutes at $500 \times g$. After washing, 350 μL of NP-40 lysis buffer was added to cell pellet and incubated on ice for 10 minutes followed by centrifugation for 12 minutes at 10,000 rpm. The supernatant and cell pellet were collected and dissolved in diethyl ether for cytosol and membrane content analysis, respectively. Samples were evacuated with nitrogen gas, capped and crimped. The sample was loaded in the Varian 300 GC-MS. The samples were analyzed by a Varian model 450 gas chromatograph coupled with a Varian model 300-MS quadruple Mass spectrometer. The column was a factor four capillary column (VF-5 ms, 30 m \times 0.25 mm ID, DF = 0.25 μm). Helium was used as the carrier gas with a flow rate of 1.0 mL/min. Samples were introduced through a split mode method. This involved a one in ten split by a Varian 450 autosampler, with a high temperature injection port (280 °C). The oven temperature was initially 50 °C for 1 minute and increased at a rate of 10 °C/min until a final temperature of 280 °C was reached for a 6 minute duration. Electrospray ionization conditions were used with

ionization energy of 70 eV. The scan range was from 70 – 600 amu. Cells exposed to LA were analyzed for both membrane and cytosolic fractional content.

1.14 Statistics

Data were presented as mean \pm SEM ($n \geq 3$) and analyzed for statistical differences using ANOVA, with $p < 0.05$ considered significant. If a difference was detected a Dunnett's or Bonferroni test was performed to compare means to a control or all means to each other, respectively ($p < 0.05$).

Results

Effect of H₂O₂ on viability of A549 cells. Initially, a concentration and time response of A549 cells to H₂O₂ was carried out to determine the appropriate range of H₂O₂ concentrations for subsequent experiments. Viability of H₂O₂-challenged A549 cells decreased in both a concentration- and time-dependent manner relative to control cells as determined by the MTT colorimetric assay (Figure 1). Significant cell death was observed following challenge with as low as 0.05 mM H₂O₂ for 6 hours, approximately 50 % of cells were dead using 0.32 mM H₂O₂ with the greatest number of cells dying following 1.5 mM H₂O₂ exposure (Figure 1 A). To investigate the progression of cell death overtime, 0.20 mM H₂O₂ was used (Figure 1 B). Following a 6 hour incubation with 0.20 mM H₂O₂, cells experienced a 15% increase of cell death, 50 % after about 10 hours, and the greatest number of cells dying at 20 and 24 hours.

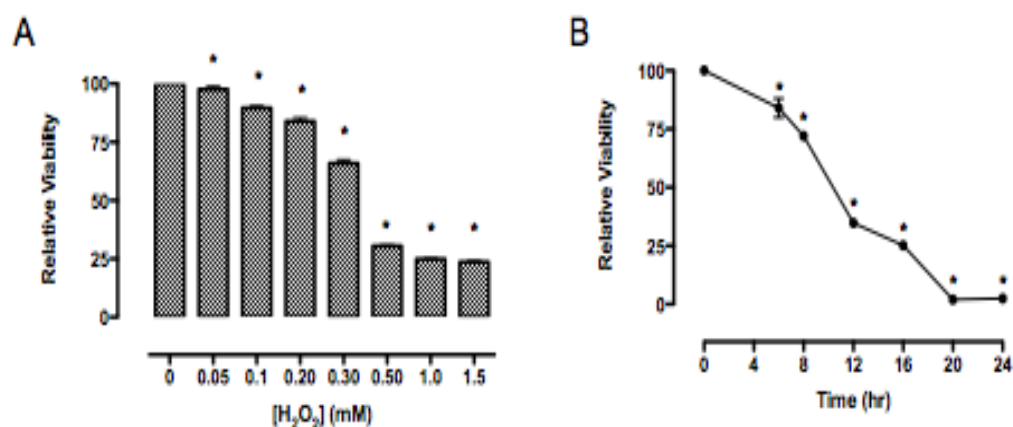


Figure 1: Effect of H₂O₂ concentration and time of exposure on the viability of A549 cells. The activity of mitochondrial dehydrogenases, using the MTT colorimetric assay, was used to assess the viability of cells challenged with H₂O₂. Cells seeded into 96-well at 10,000 cells/well and grown to 80% confluence were challenged with (A) increasing concentrations of H₂O₂ for 6 hours or (B) with 0.20 mM H₂O₂ for various time points up to 24 hours. Cells were incubated for 4 hours with MTT reagent post-H₂O₂ challenge and absorbance was measured spectrophotometrically at 570nm. Viability of H₂O₂-challenged cells was assessed relative to control cells. Data points represent mean \pm S.E.M. of 4 independent experiments performed in duplicate. * denotes a significant difference relative to control ($p < 0.05$).

Effect of PQ on viability of A549 cells. A concentration and time response of A549 cells to PQ was carried out to determine an appropriate concentration range of PQ for subsequent experiments. Viability of PQ-challenged A549 cells decreased in both a concentration- and time-dependent manner relative to control cells, determined by the MTT colorimetric assay (Figure 2). A significant decrease in cell viability was observed with as low as 0.25 mM PQ exposure for 6 hours and approximately 50 % of cell death was observed in cells exposed to 5 mM PQ (Figure 2 A). The progression of cell death over-time was examined using 1.0 mM PQ (Figure 2 B). Significant cell death was observed following a 1 hour incubation with 1 mM PQ (15%), approximately 50 % after 8 hours, with the greatest number of cells dying at 24 hour post-PQ challenge.

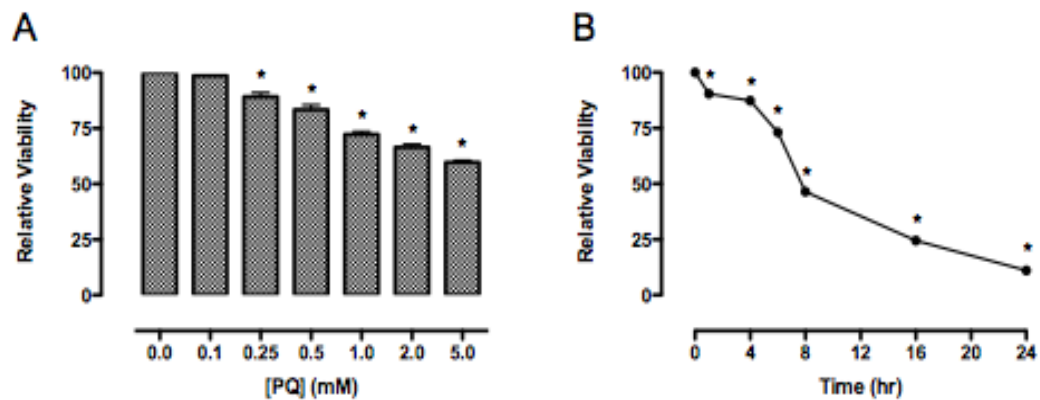


Figure 2: Effect of PQ concentration and time of exposure on the viability of A549 cells. Viability of cells challenged with PQ was assessed using the MTT colorimetric assay. Cells seeded into 96-well at 10,000 cells/well and grown to 80% confluence were challenged with (A) increasing concentrations of PQ for 6 hours or (B) with 1.0 mM PQ for various time points up to 24 hours. Following PQ challenge, cells were incubated for 4 hours with MTT reagent and the absorbance was measured spectrophotometrically at 570 nm. Viability of PQ-challenged cells was assessed relative to control cells. Data points represent mean \pm S.E.M. of 3 independent experiments performed in duplicate. * denotes a significant difference relative to control ($p < 0.05$).

Effect of LA alone on viability in A549 cells. A significant increase in cell viability of A549 cells was observed with concentrations ranging from 0.1 – 1.2 mM LA. (Figure 3).

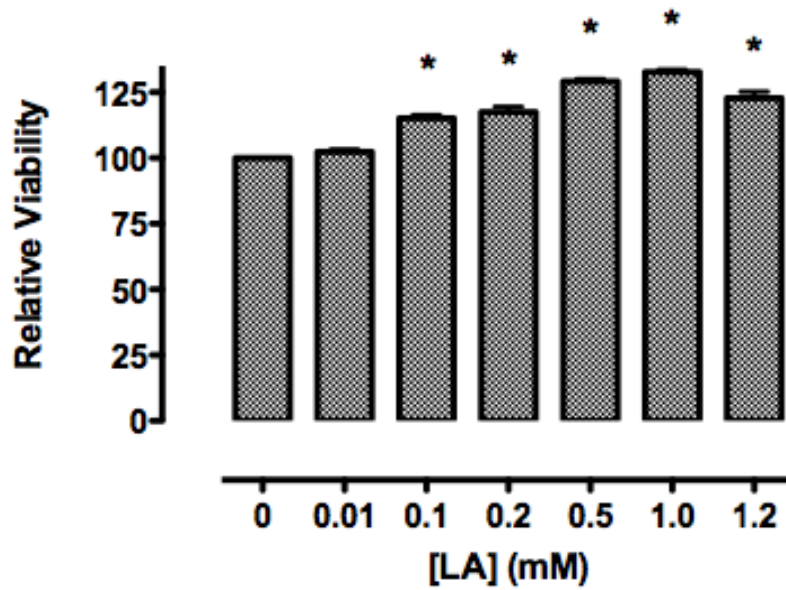


Figure 3: Effect of LA concentration on the viability of A549 cells. Viability of cells treated for 6 hours with increasing concentrations of LA were assessed using the MTT colorimetric assay. Cells were seeded into 96-well plates at 10,000 cells/well and grown to 80% confluence prior to treatment. Cells were incubated for 4 hours with MTT reagent post-LA treatment and absorbance was measured spectrophotometrically at 570nm. Viability of treated cells was assessed relative to control cells. Bars represent mean \pm S.E.M. of 3 independent experiments performed in duplicate. * denotes a significant difference relative to control ($p < 0.05$).

Effect of LA on cell viability of H₂O₂- or PQ-challenged cells. Co-treatment of LA and H₂O₂ for 6 hours resulted in a further, significant decrease in cell viability relative to cells with no co-treatment of LA (Figure 4 A). Additionally, a number of LA pre-treatment times and concentrations were also assessed with H₂O₂-challenged cells that resulted in no significant increase of A549 cellular viability (data not shown). Co-treatment of A549 cells with LA and PQ resulted in a significant increase in cell viability relative to control cells. Cells challenged with 1.0, 2.0 or 5.0 mM PQ and treated with 0.5 mM LA resulted in 31%, 28% or 22% increase in cell viability, respectively. In addition, cells challenged with 1.0, 2.0 or 5.0 mM PQ and treated with 1.0 mM LA resulted in a 40%, 37% or 32% increase of cell viability, respectively.

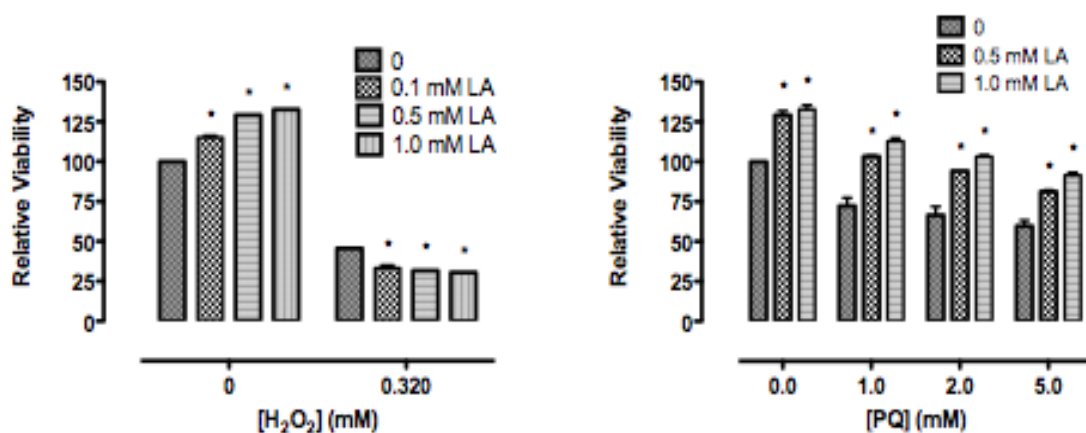


Figure 4: Effect of LA treatment on cell viability of H_2O_2 - or PQ-challenged cells.

The viability of cells co-treated with LA (0, 0.1, 0.5 or 1.0 mM) and H_2O_2 (0, or 0.320 mM) or PQ (0.5, 1.0, 2.0 or 5.0 mM) for 6 hours was assessed using the MTT colorimetric assay. Viability of challenged cells was assessed relative to untreated control cells. Bars represent mean \pm S.E.M. of 3 independent experiments performed in duplicate. * denotes a significant difference relative to cells with no co-treatment.

Effect of PQ exposure on mitochondrial membrane potential. Flow cytometric analysis of PQ-challenged cells stained with the cell permeable JC-1 dye revealed that PQ depolarizes the mitochondrial membrane as indicated by decreases in FL2-H fluorescence (Figure 5). This effect of PQ on A549 cells was concentration-dependent, reaching approximately 40, 50 or 65 % less fluorescence in cells challenged with 1.0, 2.0 or 5.0 mM PQ, respectively relative to control after 6 h post-PQ treatment.. The representative histogram (Fig. 5) shows a decrease in FL2-H fluorescence intensity with increasing PQ concentrations relative to control cells.

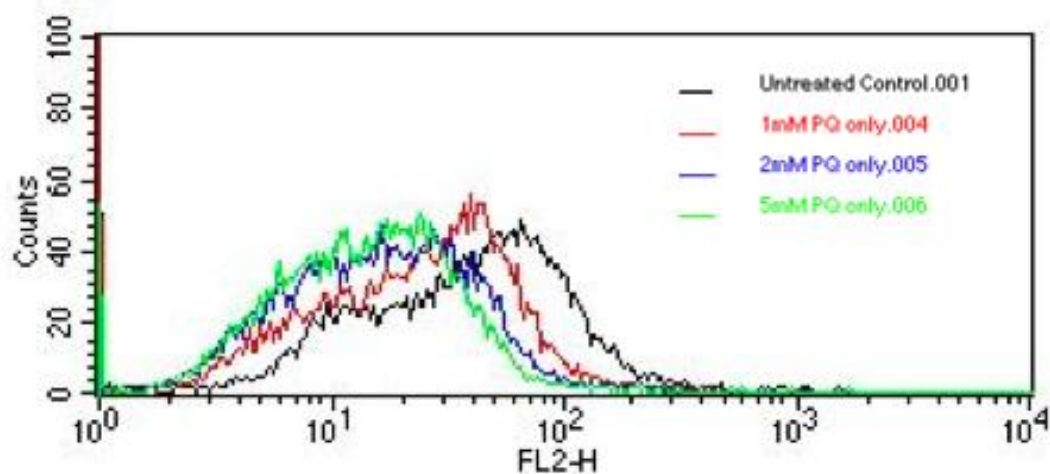


Figure 5: Representative histogram showing the effect of PQ challenge on cellular mitochondrial membrane potential. Cells seeded into 6-well plates at 0.5×10^6 cells/well and challenged with 0, 1.0, 2.0 or 5.0 mM PQ for 6 hours were stained with the membrane-permeable JC-1 fluorescent dye for 30 minutes. Membrane depolarization was represented as a decrease in red fluorescence measured on the FL2-H channel.

Effect of LA on cellular mitochondrial membrane potential in PQ-challenged cells.

Cells treated with 0.1 mM LA for 6 hours had no significant effect on mitochondrial membrane potential; however, cells treated with 0.5 mM or 1.0 mM LA resulted in significant (40% or 55%, respectively) depolarization of the mitochondrial membrane.

Co-treatment of A549 cells with 0.1, 0.5 or 1.0 mM LA had no significant preventative or augmentative effect on mitochondrial membrane depolarization in either 2.0 or 5.0 mM PQ-challenged cells (Figure 6).

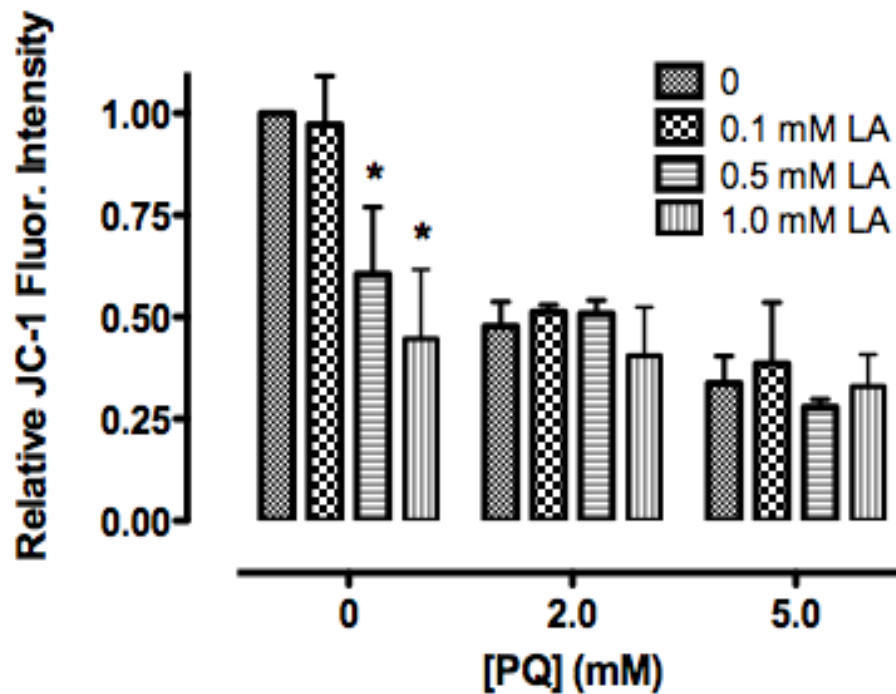


Figure 6: Effect of LA treatment on mitochondrial membrane potential in PQ challenged A549 cells. Cells were co-treated with LA (0, 0.1, 0.5 or 1.0 mM) and/or PQ (0, 2.0 or 5.0 mM) for 6 hours. Following treatment cells were stained for 30 minutes with cell permeable JC-1 fluorescent dye and analyzed using flow cytometry on the FL2-H channel. Mitochondrial membrane depolarization was indicated by a decrease in red fluorescence (FL2-H) intensity. Bars represent mean \pm S.E.M. of 4 independent experiments. * denotes a significant differences relative to cells with no co-treatment.

Effects of LA on lipid peroxidation in PQ challenged A549 cells Since it is known that one of the primary actions of PQ is to generate ROS via redox cycling, potentially causing damage to PUFAs, the role of lipid peroxidation in PQ-induced toxicity of A549 cells was assessed. Spectrophotometric analysis of PQ-challenged cells stained with N-methyl-2-phenylindole revealed no significant effect on lipid peroxidation. Similarly, no significant evidence of lipid peroxidation was revealed when cells were treated with 0.1 or 0.5 mM LA alone or with 5.0 mM PQ-challenged cells for 6 hours (Figure 7).

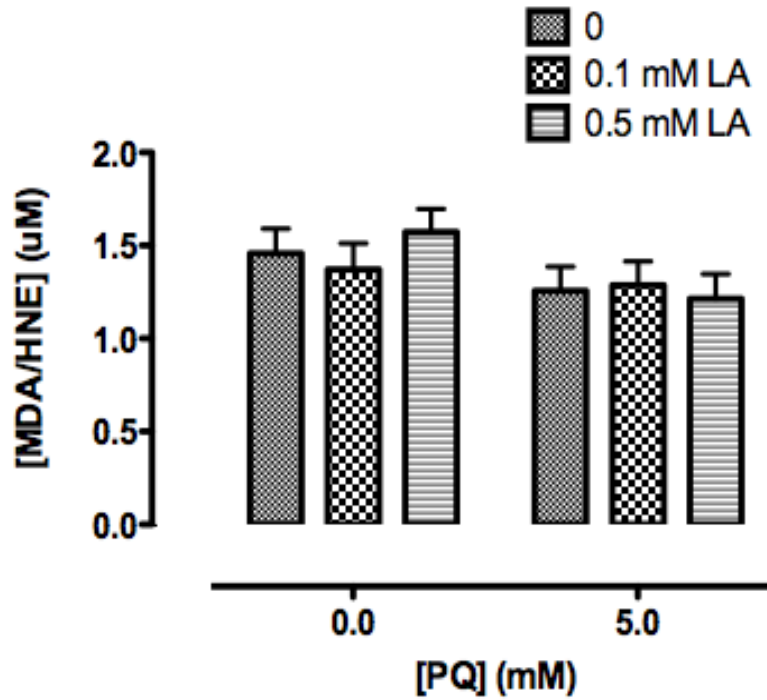


Figure 7: Effect of LA treatment on lipid peroxidation in PQ challenged A549 cells.

Following a 6 hour co-treatment with LA (0.1 or 0.5 mM) and/or PQ (0 or 5.0 mM), cells were lysed and incubated with the chromogenic reagent, N-methyl-2-phenylindole. Lipid peroxidation by-products, malondialdehyde (MDA) and 4-hydroxylalkenal (HNE), react with the chromogenic reagent to form stable chromophores that can be detected spectrophotometrically at 585 nm. Bars represent mean \pm S.E.M. of 3 independent trials.

Effect of PQ on DNA fragmentation in A549 cells. Flow cytometric analysis of PQ-challenged cells stained with AlexaFluor*488 anti-BrdU antibody revealed that PQ does cause DNA fragmentation indicated by an increase in FL1-H fluorescence intensity relative to control cells (Figure 8 A-C). Treatment of A549 cells with 2.0 or 5.0 mM PQ resulted in a 1.69- and 1.74-fold increase in DNA fragmentation relative to control cells (Figure 8 D).

Effect of LA on DNA fragmentation of PQ-challenged A549 cells.

Flow cytometric analysis of cells treated with LA (0.1, 0.5 or 1.0 mM) for 6 hours revealed no significant increase of DNA fragmentation as indicated by no statistically significant increase in FL1-H fluorescence (Figure 8 D, O LA). However, co-treatment of cells with LA (0.1, 0.5 or 1.0 mM) and PQ (2.0 or 5.0 mM) resulted in a concentration-dependent increase in DNA fragmentation. Cells challenged with 2.0 mM PQ and treated with 1.0 mM LA resulted in 1.22-, 1.35- or 1.81-fold increase in DNA fragmentation relative to cells with no co-treatment of LA, respectively. Furthermore, cells challenged with 5.0 mM PQ and treated with 0.1, 0.5 or 1.0 mM LA resulted in a 1.17-, 1.77- or 2.26-fold increase in DNA fragmentation relative to cells with no co-treatment of LA, respectively (Figure 8 D).

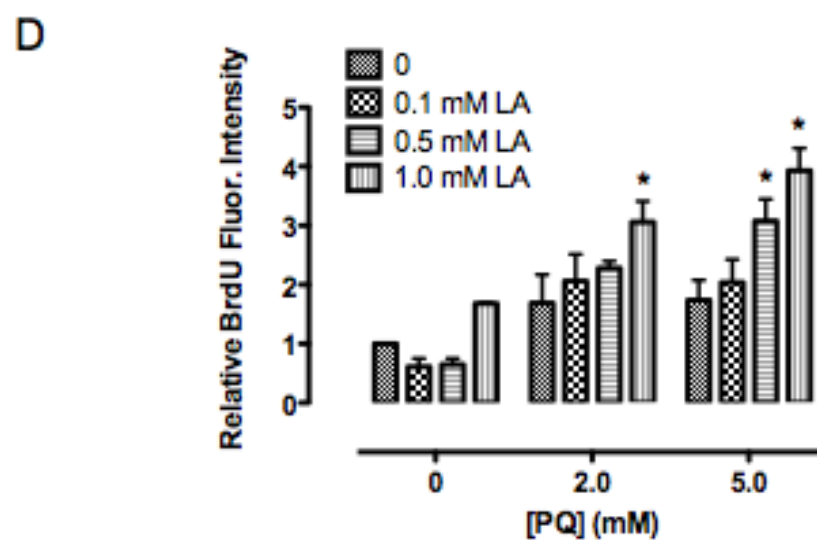
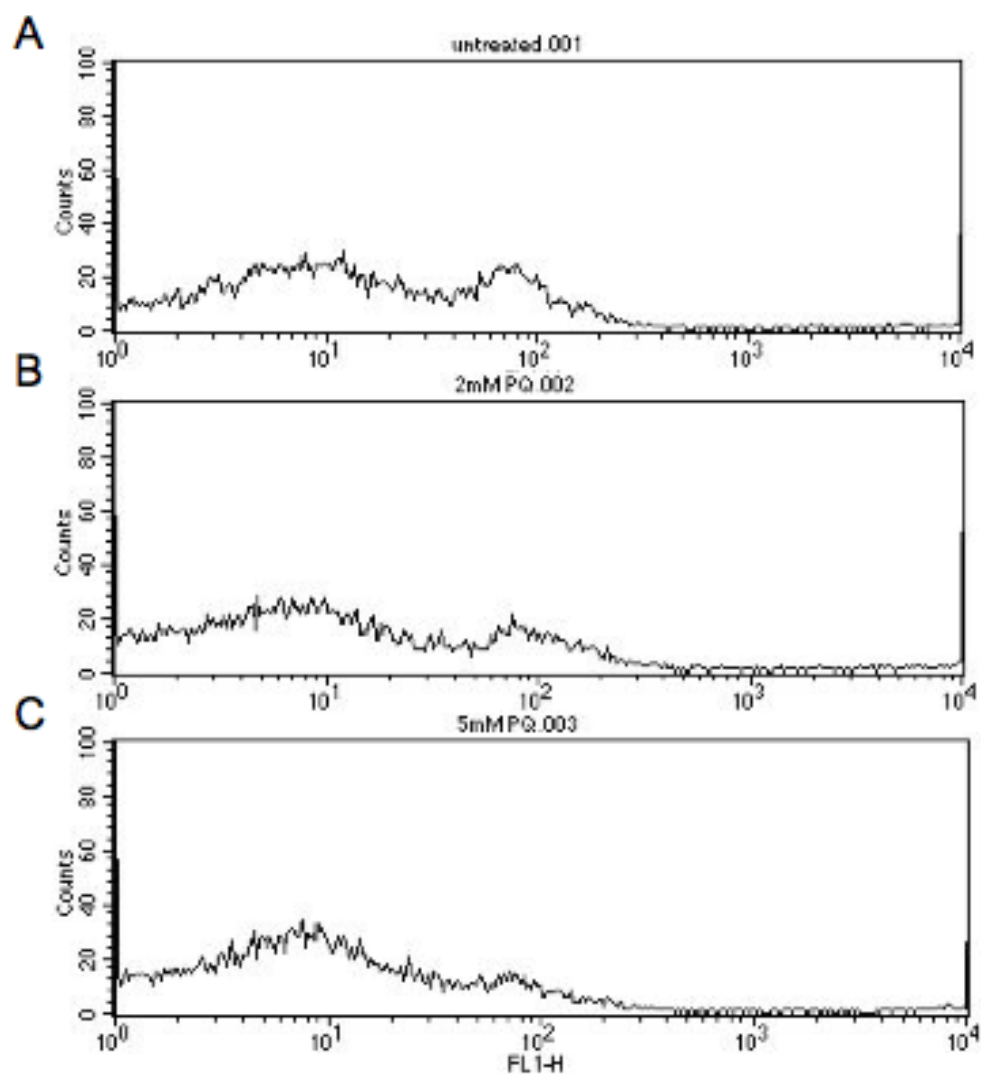


Figure 8: Effect of LA treatment on DNA fragmentation in PQ challenged A549

cells. Cells were seeded into 6-well plates at 1.0×10^6 cells/well and treated with LA (0, 0.1, 0.5 or 1.0 mM) and/or PQ (0, 2.0, or 5.0 mM) for 6 hours and fixed overnight. Using immunohistochemical techniques DNA fragments were stained with AlexaFluor*488 dye-labeled anti-BrdU antibody and analyzed using flow cytometry on the FL1-H channel. DNA fragmentation was indicated by an increase in green fluorescence (FL1-H) intensity caused by the incorporation of the anti-BrdU antibody into DNA break sites. Representative histograms (A-C) show FL1-H fluorescence intensity of stained cells following 0 (A), 2 (B) and 5 mM (C) PQ challenge for 6 hours. Bars represent mean \pm S.E.M. of 3 independent trials (D). * denotes significant difference relative to cells with no co-treatment ($p < 0.05$).

Effect of PQ and LA exposure on total nuclear DNA content. Flow cytometric analysis of PQ-challenged cells stained with propidium iodide revealed no significant increase of sub-G1 DNA content in A549 cells challenged with 5.0 mM PQ for 6 hours relative to control cells (Figure 9 A). Representative flow cytometric histograms show a slight but non-significant tailing of the G0/G1 peak that is characteristic of DNA fragmentation (Figure 9 C). In A549 cells treated with 0.1, 0.5 or 1.0 mM LA for 6 hours, no significant changes to the cell cycle profile or indications of DNA fragmentation were observed (data not shown).

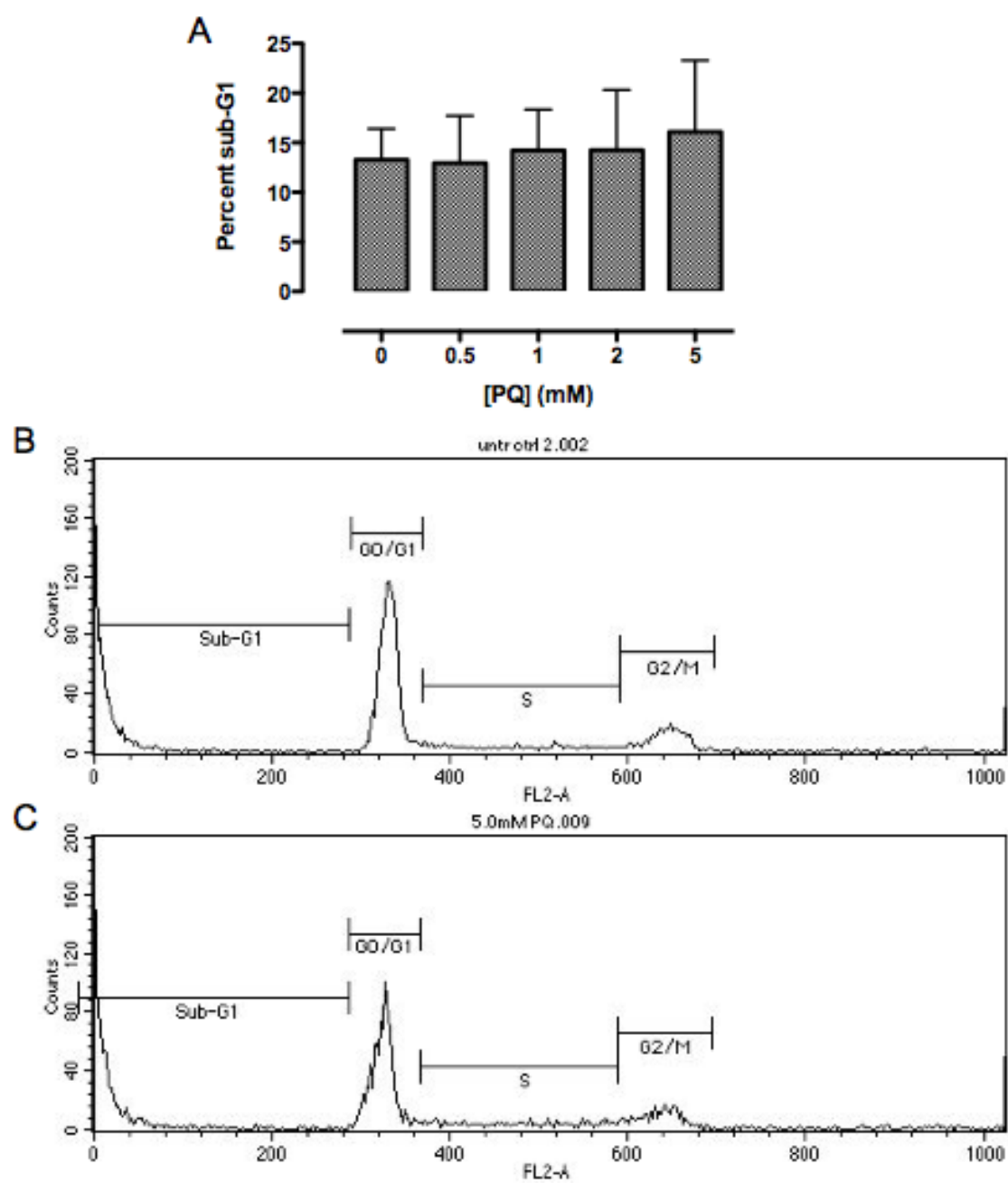


Figure 9: Effect of PQ challenge on DNA cell cycle of A549 cells. Following 0, 0.5, 1.0, 2.0 or 5.0 mM PQ challenge for 6 hours cells were stained for 30 minutes with propidium iodide (PI) for flow cytometric analysis of total nuclear DNA content and the percentage of cells in each phase of the cell cycle. PI fluorescence was measured on the FL2-A channel where DNA fragmentation was indicated by a sub-G1 peak representing the hypodiploid DNA. Representative histograms show FL2-A fluorescence intensity of stained cells following 0 mM (B) and 5 mM (C) PQ exposure. Bars represent mean \pm S.E.M. of 3 independent trials.

Effect of PQ and LA exposure on apoptosis in A549 cells. Flow cytometric analysis of PQ-challenged cells dually stained with annexin V and propidium iodide dye revealed that PQ exposure does not result in any significant apoptotic features, indicated by phosphatidylserine translocation, when challenged with 1.0, 2.0 or 5.0 mM PQ for 6 hours (Figure 10 A). Representative flow cytometric dot-plots show no apparent changes in apoptotic (LR) and/or necrotic (UL & UR) cells following 0, 2.0 or 5.0 mM PQ challenge for 6 hours (Figures 10 B-D). Similarly, LA treated cells did not show any significant apoptotic features either when treated with 0.1, 0.5 or 1.0 mM LA for 6 hours (data not shown).

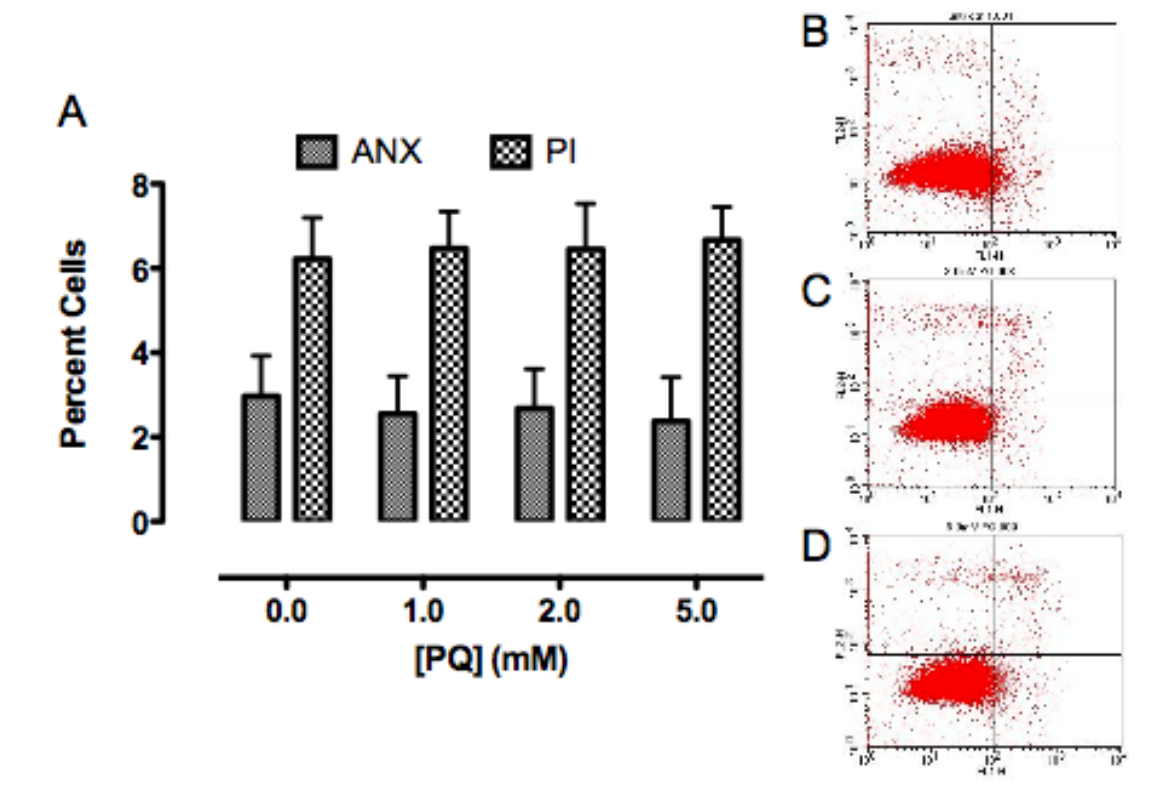


Figure 10: Effect of PQ challenge on apoptosis of A549 cells. Following 0, 1.0, 2.0 or 5.0 mM PQ challenge for 6 hours, cells were suspended via trypsinization and dually stained for 15 minutes with annexin V (ANX) and propidium iodide (PI) for flow cytometric analysis of phosphatidylserine translocation, an indicator of apoptosis. Representative flow cytometric dot-plots show similar ANX and PI staining among 0, 2.0 and 5.0 mM PQ-challenged cells (6 hour exposure B-D, respectively). Annexin V and PI fluorescences were measured on FL1-H and FL2-H channels, respectively. ANX⁻/PI⁻ cells were considered live (LL), ANX⁺/PI⁻ cells apoptotic (LR) and ANX⁺/PI⁺ cells necrotic (UL & LL). Bars represent mean \pm S.E.M. of 3 independent trials (A). LL: lower left; LR: lower right; UL: upper left; UR: upper right.

Effects of LA on intracellular calcium in PQ-challenged A549 cells. Flow cytometric analysis of cells treated with LA (0.1, 0.5 or 1.0 mM) for 6 hours revealed no significant increase in intracellular Ca^{2+} as indicated by no increase in FL1-H fluorescence (Figure 12). A549 cells treated with 1.0, 2.0 or 5.0 mM PQ resulted in a 1.28-, 1.46- or 1.61- fold increase of FL1-H fluorescence, respectively (Figure 11). Co-treatment of cells with LA (0.1, 0.5, or 1.0 mM) and PQ (2.0, 5.0 mM) resulted in a concentration-dependent increase in intracellular Ca^{2+} . Cells challenged with 2.0 mM PQ and treated with 0.5 or 1.0 mM LA resulted in a 1.17- or 1.50-fold increase in intracellular Ca^{2+} relative to cells with no co-treatment of LA, respectively. Furthermore, cells challenged with 5.0 mM PQ and treated with 0.1, 0.5 or 1.0 mM LA resulted in a 1.42-, 1.56-, or 2.10-fold increase in intracellular Ca^{2+} relative to cells with no co-treatment of LA, respectively (Figure 12).

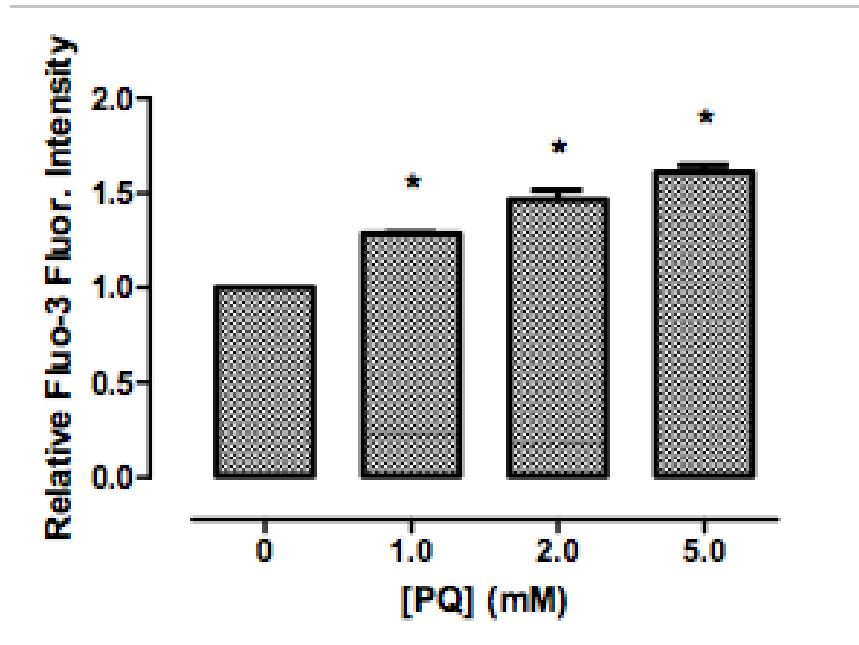


Figure 11: Effect of intracellular Ca^{2+} levels in PQ-challenged A549 cells. Following challenge with 0, 1.0, 2.0 or 5.0 mM PQ for 6 hours, cells were stained with Fluo-3AM and analyzed using flow cytometry (minimum 15,000 events; FL1-H). The rise in relative fluorescence indicates an increase of intracellular Ca^{2+} levels compared to control cells. Bars represent mean \pm S.E.M. of 3 independent trials. * denotes a significant difference relative to control ($p < 0.05$)

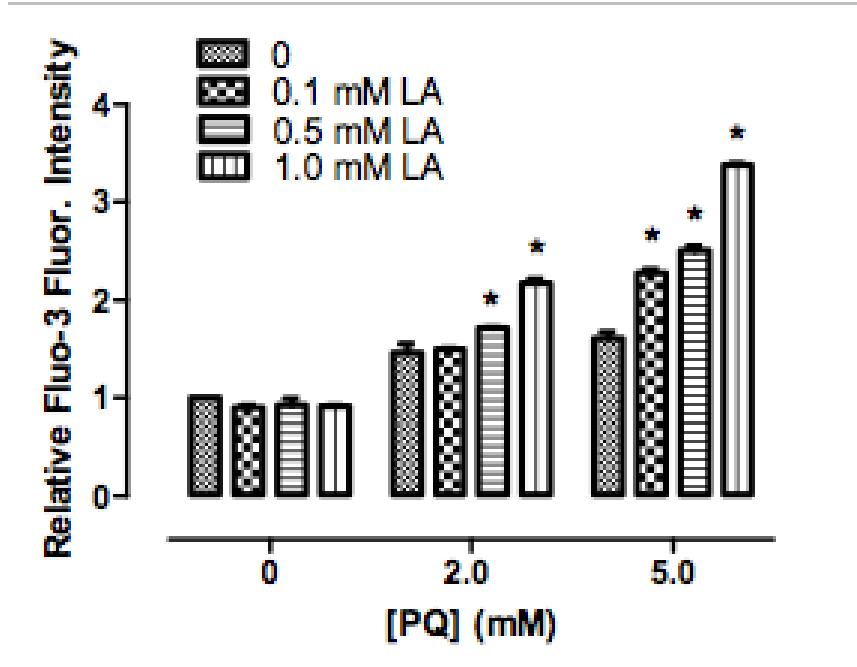


Figure 12: Effect of LA treatment on intracellular Ca^{2+} levels in PQ-challenged A549 cells. Cells were co-treated with LA (0, 0.1, 0.5 or 1.0 mM) and/or PQ (0, 2.0 or 5.0 mM) for 6 hours. Following treatment cells were stained for 45 minutes with Fluo-3 and analyzed flow cytometrically using the FL1-H channel. A rise of intracellular Ca^{2+} levels was indicated by the increase on green fluorescence (FL1-H) intensity. Bars represent mean \pm S.E.M. of 3 independent experiments. * denotes a significant difference relative to cells with no co-treatment ($p < 0.05$).

GC/MS analysis of the cytosolic and membrane isolate fractions of cells exposed to LA.

A standard curve was established using specific LA concentrations, from which a linear regression analysis was conducted. The slope of the line is 77736, and the intercept is -831760. Cells exposed to 0.5 mM LA for 6 hours, detected the LA peak in only the membrane isolate fraction, which had an area of 2203401 ± 5403 units equivalent to be 39.04 μ M LA.

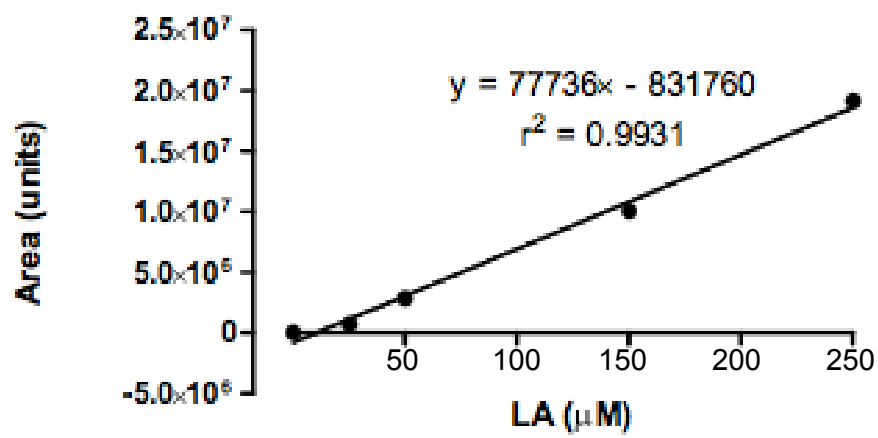


Figure 13: Standard curve for the quantification of LA in the cell membrane isolate by GC/MS: The abscissas are the concentration (μM) and the ordinate is the area of the resulting GC peak. The line is drawn using linear regression analysis. The slope of the line is 77,736 and the intercept is -831,760.

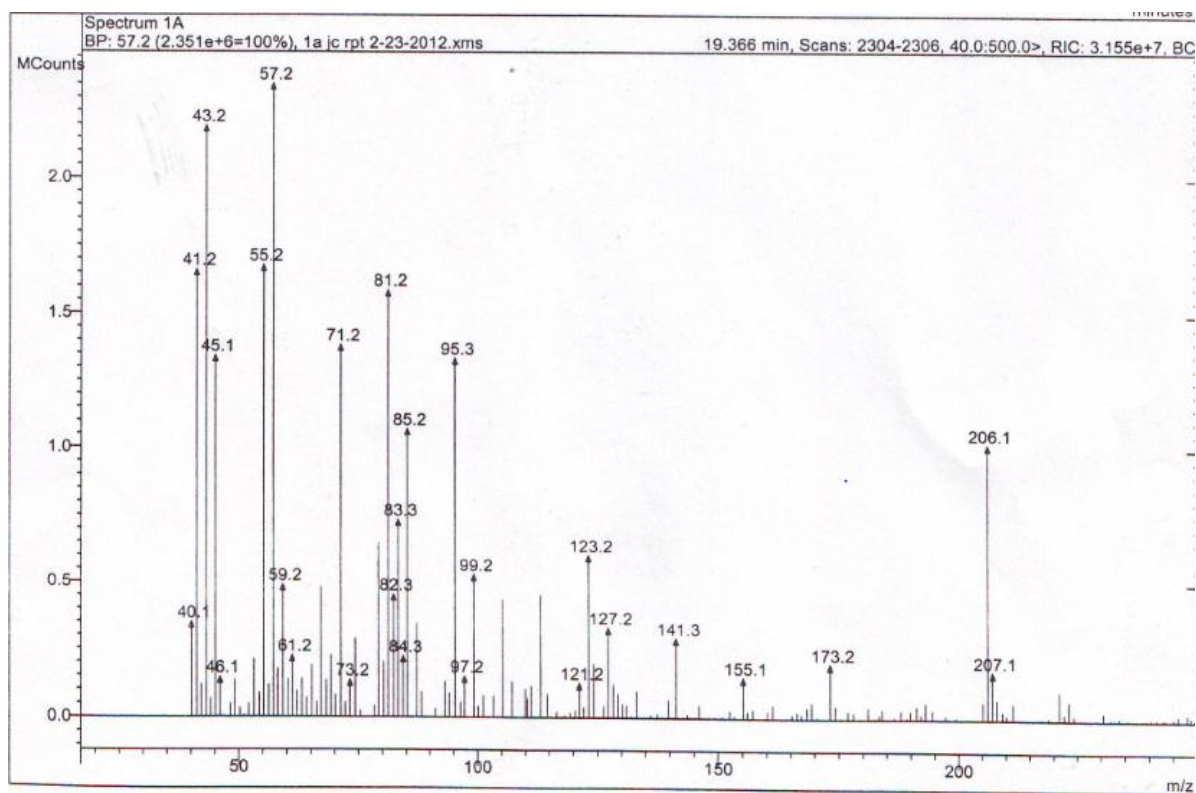


Figure 14: Identification of a sample peak as LA: A549 cells were treated with 0.5 mM LA for 6 hours. After treatment cells were harvested and lysed for cell membrane content analysis of LA. We were able to detect LA in the cellular membrane based on the pattern of fragmentation in comparison with the MS library.

Discussion

H₂O₂ is a cell permeable molecule having both toxicological and physiological signaling roles. It can be generated by oxidoreductases, the dismutation of the superoxide radical, electron leakage from the mitochondrial electron transport chain and through redox cycling of quinines and flavoproteins (Forman, 2007). It can become further reduced by one of three pathways: (1) catalyzed by catalase into water and oxygen, (2) detoxified by glutathione peroxidase in the presence of GSH to form water and GSSG and (3) in the presence of either the superoxide radical or a transition metal it will produce the hydroxyl radical (Klaassen, 2001). The hydroxyl radical is capable of reacting with proteins, DNA or PUFA's causing cellular damage and/or cell death. Also, H₂O₂ can activate Ras (Chetsawang *et al.*, 2010; Janssen *et al.*, 1997), NF-κβ and protein tyrosine phosphatase (PTP) signaling pathways that activate an array of genes involved in inflammation, angiogenesis and apoptosis (Allen & Tresini, 2000; Forman, 2007). Due to the central role of H₂O₂ in free radical formation, it has been used as a model for oxidative stress-induced injuries. In this study, the mechanism(s) responsible for the H₂O₂-induced toxicity in A549 lung epithelial cells were investigated *in vitro*.

A549 cells challenged with H₂O₂ resulted in both concentration- and time-dependent decreases in cell viability as measured by the MTT assay (Figure 1). In our studies, A549 cells exposed to 0.5 mM H₂O₂ for only 6 hours resulted in approximately 75% decrease in cell viability. In another study, A549 lung epithelial cells exposed to 0.5 mM, 1.0 mM or 2.0 mM H₂O₂ for 7 hours resulted in a 20%, 25% and 35% decrease in

cell viability (Arsalane *et al.*, 1997). The discrepancy between these studies could be attributed to the differences between the density of cells in the plate wells, in our case having 5 times less cells per well. H₂O₂ has been reported to exhibit variable cytotoxicity depending on cell type and density. Whittemore *et al.* (1995) used a mix of primary rat cortical neurons and exposed cells for 3, 6 and 12 hours to 100 µM H₂O₂ that resulted in a 55%, 75% and 88% decrease in cell viability, respectively. This study also exposed high and low density cell cultures to 30 µM or 100 µM H₂O₂ for 24 hours. High-density cell cultures resulted in a 5% and 60% decrease in viability, while low-density cell cultures resulted in a 45% and 75% decrease in cell viability after being exposed to 30 µM or 100 µM H₂O₂, respectively.

The protective effect of the amphipathic, di-thiol antioxidant LA was investigated in H₂O₂-induced cytotoxicity. Due to its low redox potential, the LA/DHLA system not only has a high reactivity to free radicals, but is also capable of regenerating ascorbic acid and α -tocopherol, elevating intracellular GSH content and modulating transcription factor activity (Bilska *et al.*, 2005). These four properties make LA a suitable candidate for the amelioration of H₂O₂-induced toxicity.

In order to examine the protective effects of LA against oxidant-induced toxicity, the effect of different concentrations of LA on A549 cells at different exposure times was investigated. Generally, the chemical and physical characteristics of a drug as well as the concentration levels determine its antioxidant properties. It has been reported that that LA and DHLA, based on their characteristics, concentrations, and experimental cell type can

act as an antioxidant or a prooxidant *in vitro* (Çakatay, 2006; Biewenga *et al.*, 1997). Most studies have shown that a pre-incubation of human fibroblasts, neuroblastoma cells, rat cortical neurons or rat cardiac cells for 24-48 hours with LA (0.05-200 μ M) have resulted in an increase of cell viability (Jia *et al.*, 2008; Jia *et al.*, 2009; Suzuki *et al.*, 1991; Zhang *et al.*, 2001; Cao *et al.*, 2003). In comparison, a pre-incubation of lung epithelial cells or pancreatic β cells for 24 hours with LA (0.5-1.2 mM) has resulted in a decrease of cell viability (Choi *et al.*, 2009; Wan Lee *et al.*, 2009). The results of our study showed that an incubation time of 6 hours with 0.1 mM, 0.5 mM or 1.0 mM LA had no negative impact on viability (Figure 3).

The effect of LA treatment on the viability of H₂O₂-challenged cells was assessed via the MTT assay. Results from previous studies have shown that preincubation of rat cortical neurons for 48 hours with 1.0, 5.0 and 10 μ M LA and then treated with 100 μ M H₂O₂ for 24 hours, resulted in a 15%, 50% and 75% increase in cell viability compared to control with no pre-treatment (Zhang *et al.*, 2001). Jia *et al.* (2008) pretreated neuroblastoma cells with 200 μ M LA for 24 hours, followed by a 24 hour incubation with 100 μ M H₂O₂ that resulted in a 35% increase in cell viability. In our studies we analyzed a combination of LA pre-incubation times (6-24 hours) with H₂O₂ treatment times (4-24 hours) and were unable to confirm a protective effect of LA in H₂O₂-induced oxidative injury in A549 cells (data not shown). Co-administration of LA and H₂O₂ to A549 cells for different time points, also did not protect against H₂O₂-induced oxidative injury (Figure 4 A).

The lack of protective effect of LA against H₂O₂ cytotoxicity cannot be explained from the results of this study. Several studies have shown that neither, LA or DHLA react with H₂O₂ at a significant rate and are therefore, are incapable of directly scavenging H₂O₂ (Biewenga *et al.*, 1997; Packer *et al.*, 1998; Scott *et al.*, 1994). Other studies have shown that LA and DHLA can indirectly protect against H₂O₂-induced toxicity through the induction of other antioxidants (GSH, ascorbate, etc.) and phase II enzymes (NQO1) (Jia *et al.*, 2008; Kagan *et al.*, 2003). However, we were unable to substantiate an antioxidant or prooxidant effect of LA in H₂O₂-induced oxidative damage, so we decided to pursue its effects on another oxidant, paraquat (PQ).

PQ undergoes a cyclic reduction-oxidation reaction that forms the superoxide radical leading to the generation of hydrogen peroxide and subsequently the hydroxyl radical (Hoet & Nemery, 2000). The polyamine uptake system found on alveolar type I and II cells selectively binds and transports PQ into the lung, resulting in destructive and proliferative phases (Autor, 1997; Heath & Smith, 1977). The destruction of the pulmonary epithelial cells results in a decrease of surfactant, water and ion transport and epithelial regeneration. The inflammatory response is stimulated during the destructive phase and maintained during the proliferative phase that involves extensive fibrosis. Fibrosis causes the loss of pulmonary elasticity, respiratory impairment, inefficient gas exchange and eventually anoxia (Hoet & Nemery, 2000). In this study, the mechanism(s) behind PQ-induced toxicity in A549 lung epithelial cells were investigated *in vitro*.

A549 lung epithelial cells challenged with PQ resulted in both concentration- and time-dependent decreases in cell viability. These results corresponded with those found by Mitsopoulos & Suntres (2010) using A549 lung epithelial cells. Mitsopoulos & Suntres (2010) showed that the decrease in cell viability was associated with a concomitant time- and concentration-dependent increase in the cellular uptake of PQ. A study by Weidauer *et al.* (2004) that used primary alveolar type II cells isolated from rat lung had comparable results, where exposure to 1.0 mM PQ for 24 hours resulted in an 80% decrease in viability.

Many lipophilic cations and dications such as PQ are taken up electrophoretically into mitochondria by direct passage through the phospholipid bilayer driven by the MMP (Cochemé & Murphy, 2008). Once in the matrix, PQ is predominantly reduced by complex I to form a PQ cation, which can react with oxygen to form the superoxide radical (Klaassen, 2001). During this process, PQ is known to inhibit the transfer of electrons along complex I, thereby inhibiting the production of ATP. A lack of ATP compromises the operation of ATP-dependent ion pumps leading to the loss of ionic and volume-regulatory controls (Buja *et al.*, 1993). ATP drives ion transporters such as the Na⁺, K⁺-ATPase in the plasma membrane, the Ca²⁺-ATPase in the plasma and endoplasmic reticulum membranes and H⁺-ATPase in the membrane of lysosomes and neurotransmitter-containing vesicles (Klaassen, 2001).

By impairing ATP synthesis, PQ also inhibits Ca²⁺ export from the cytoplasm via the Ca²⁺-ATPase transporter leading the sustained elevations of high cytosolic Ca²⁺ levels

that can directly or indirectly modulate many physiological processes (Giacomello *et al.*, 2007). High cytosolic Ca^{2+} levels cause an increase Ca^{2+} uptake into the matrix via the Ca^{2+} -uniporter, which like ATP synthase, utilizes the negative MMP as the driving force (Giacomello *et al.*, 2007; Schweizer & Richter, 1996). The uptake of Ca^{2+} into the matrix causes the dissipation of the MMP further inhibiting the flux of electrons across the electron transport chain and therefore, the synthesis of ATP (Richter & Kass, 1991). In our study, PQ challenge of A549 cells resulted in a concentration-dependent increase in intracellular calcium concentrations (Fig. 11).

Flow cytometric analysis of cellular MMP in A549 cells showed that PQ decreased cellular MMP with increasing concentrations of PQ (Figure 5). In a study by Mitsopoulos & Suntres (2011) A549 cells exposed to 1.0 mM PQ for 8 hours resulted in an approximately 50% decrease in MMP, correlating with our results. These findings were also supported by a concentration-dependent increase in intracellular Ca^{2+} levels following a 6 hour exposure to PQ (Figure 11). Intracellular Ca^{2+} activates three critical metabolic enzymes in the matrix (pyruvate, α -ketoglutarate and isocitrate dehydrogenases) that drive electrons into the electron transport chain (Giacomello *et al.*, 2007). These electrons derived from NADH and ubiquinone can directly react with oxygen or other electron acceptors, such as PQ, to generate free radicals. An increase production of ROS also causes the loss of the MMP and the simulation of mitophagy, a specialized form of autophagy that selectively eliminates defective mitochondria (Marchi *et al.*, 2012)

The effect of LA treatment on the viability of PQ-challenged cells was assessed via the MTT assay as well. PQ challenged cells (1.0, 2.0 or 5.0 mM) caused a 27%, 36% or 41% reduction in cell viability compared to an untreated control, respectively (Figure 2). The treatment of PQ-challenged A549 cells with 0.5 mM LA resulted in a 26%, 35% or 48% reduction of cell viability; while the treatment of PQ-challenged cells with 1.0 mM LA resulted in a 21%, 30% or 42% reduction of cell viability compared to the LA control (Figure 4). These data suggest that LA did not alter the PQ-induced decreases in cell viability.

The effect of LA treatment on PQ-challenged A549 cells was further investigated by flow cytometric analysis of cellular MMP. The treatment of A549 cells with LA was not effective in preventing the decreases of MMP in PQ-challenged cells (Figure 6). In fact, LA treatment alone caused a decrease in MMP, which suggests that LA does not act as an antioxidant, but most likely as a prooxidant. Flow cytometric analysis of intracellular Ca^{2+} levels suggests LA alone does not increase Ca^{2+} levels, however co-treatment of LA and PQ caused the potentiation of intracellular Ca^{2+} levels, further suggesting the prospect of prooxidant activity (Figure 12).

The precise mechanism(s) by which LA potentiated the effects of PQ on MMP and intracellular calcium levels are not understood at this time. Studies have shown that LA is capable of decreasing the MMP through the stimulated release of Ca^{2+} in rat liver mitochondria (Schweizer & Richter, 1996) and through the promotion of the mitochondrial permeability transition (MPT) in permeabilized hepatocytes and rat liver

mitochondria (Saris *et al.*, 1998; Morkunaite-Haimi *et al.*, 2003). MPT is an abrupt increase in the mitochondrial inner-membrane permeability caused by opening of a proteinaceous pore that spans both mitochondrial membranes (Lemasters *et al.*, 1998; Kroemer *et al.*, 1998). MPT pore opening is stimulated by many factors that include the accumulation of Ca^{2+} and by the oxidation of pyridine nucleotides and thiols in the pore. Stimulating MPT permits a free influx of protons into the matrix space, causing the dissipation of the MMP.

LA is also capable of directly oxidizing critical thiol groups in proteins rendering them inactive (Moini *et al.*, 2002). Adenine nucleotide translocase (ANT) is a mitochondrial carrier that forms the inner membrane channel of the mitochondrial permeability transition pore (mPTP). Oxidation of thiol residues on ANT induces opening of the mPTP. Specifically, the oxidation of Cys⁵⁶ alters the conformation of ANT in such a way that it mimics the effect of Ca^{2+} , which converts ANT into a non-specific pore, leading to the dissipation of the MMP. Also, a study by Saris *et al.* (1998) revealed that low concentrations of DHLA (0.01-0.1 mM) caused the oxidation of pyridine nucleotides. This suggests an increased production of ROS and exhaustion of the mitochondrial antioxidant capacity, which can lead to the loss of the MMP.

It should be noted that overproduction of ROS and RNS can also be secondary to the intracellular hypercalcemia, as Ca^{2+} activates enzymes that generate ROS and/or RNS. Ca^{2+} accelerates the hydrogen output from the citrate cycle by activating dehydrogenases and, as a result, the flux of electrons along the electron transport chain

increasing the likelihood of ROS formation (Klaassen, 2001; Cochemé & Murphy, 2008). Also, Ca^{2+} activates proteases that proteolytically convert xanthine dehydrogenase into xanthine oxidase, whose byproducts are the superoxide radical and hydrogen peroxide which, can further cause damage to the cell (Klaassen, 2001).

Lipid peroxidation is one of the major outcomes of free radical-mediated injury to tissue and can greatly alter the physicochemical properties of membrane lipid bilayers. Thus, the effect of PQ on lipid peroxidation was also investigated. A549 cells challenged with PQ for 6 hours resulted in no significant changes in the formation of the reactive carbonyl compounds (MDA/HNE), corresponding with other studies (Kim *et al* 2011; Weidauer *et al* 2004). In mouse liver homogenate, Peter *et al* (1992) found that 2.5 mM PQ significantly induced lipid peroxidation, but was not the primary cause of PQ-induced cell death. No significant changes were indicated in the assessment of lipid peroxidation in cells treated with LA only for 6 hours, correlating with similar findings in LA treated rat lung homogenate (Goraca & Jozefowicz-Okonkwo 2007). Together, LA and PQ treatment also had no effect on lipid peroxidation (Figure 7). GC/MS analysis of the cytosolic and membrane fractions of cells exposed to LA showed the presence of LA only with the membrane fractions (Figure 13), suggesting that LA might stabilize the cell membranes or ameliorate the toxic effects of ROS generated from PQ metabolism.

The increase of Ca^{2+} levels, along with the decrease in MMP, the generation of ROS and/or RNS and the depletion of ATP can induce necrosis and/or trigger controlled pathways of apoptotic cell death (Choi *et al* 2009; Lemasters *et al.*, 1998; Kroemer *et al.*,

1998). DNA damage is a hallmark of apoptotic cell death that occurs in response to various apoptotic stimuli. Using standard immunohistochemical techniques employed by the TUNEL assay, flow cytometric analysis revealed that A549 cells challenged with PQ for 6 hours resulted in a concentration-dependent increase of DNA fragmentation (Figure 8 A-C). In apoptosis, a portion of DNA is lost from the cell through the loss of apoptotic bodies and extensive DNA fragmentation, which can be identified by their fractional DNA content (Darzynkiewicz *et al.*, 2001). Flow cytometric analysis revealed that A549 cells challenged with PQ (0.5, 1.0, 2.0 or 5.0 mM) for 6 hours had no effect on the DNA cell cycle profile. This suggests that the PQ-induced DNA fragmentation was not substantial enough to produce a sub-G₁ peak or block entry into the S-phase of the cell cycle. In a study by Takeyama *et al.* (2004) human lung epithelial-like cells challenged with 1.0 mM PQ for 8 hours resulted in a significant increase of DNA fragmentation assessed via TUNEL assay. They also determined that cells challenged with 10 mM PQ for 3 hours significantly blocked the entry into the S-phase of the cell cycle. In a different study, Li & Sun (1999) challenged PC12 cells with 0.5 mM PQ for 24 hours resulting in “ladder-like” patterns and TUNEL positive cells indicative of DNA fragmentation. Our approach may not have been severe enough to cause changes in the cell cycle patterns after 6 hours.

It has been shown that DNA damage is associated with an increase of intracellular Ca²⁺ and ROS (Choi *et al.*, 2009). Our study showed PQ induced a concentration-dependent increase in intracellular Ca²⁺ and DNA fragmentation. In addition to its ability to facilitate the formation of ROS and RNS, an increase of intracellular Ca²⁺ can also

stimulate the hyperactivation and upregulation of poly(ADP-ribose)polymerase (PARP-1) and translocation of apoptosis-inducing factor AIF into the nucleus (Choi *et al.*, 2009). PARP-1 is a nuclear enzyme that responds to DNA damage and facilitates DNA repair (Hong *et al.*, 2004; Yu *et al.*, 2002). Minimal DNA damage activates the DNA repair machinery, while severe insults that result in extensive DNA damage will induce PARP-1 over-activation and cell death (Hong *et al.*, 2004). AIF is a mitochondrial flavoprotein that upon PARP-1 activation will translocate into the nucleus and mediate chromatin condensation and large-scale fragmentation of DNA (Hong *et al.*, 2004; Yu *et al.*, 2002). AIF translocation occurs quickly after PARP-1 activation and precedes cytochrome c release and caspase activation.

The effect of LA treatment of PQ-challenged A549 cells on DNA damage was also assessed by DNA profiles and the TUNEL assay. Flow cytometric analysis of DNA content revealed no significant changes in the cell cycle profiles of LA treated cells with or without PQ-challenge. Further investigation of DNA damage by the TUNEL revealed LA treatment alone did not increase DNA damage, however, LA treatment of PQ-challenged cells resulted in the potentiation of PQ-induced DNA fragmentation. This was further supported in our studies through the potentiation of intracellular Ca^{2+} concentrations of PQ-challenged cells by LA treatment. Our experiments have determined the presence of LA was detected only in the membrane fraction and in addition, LA alone is capable of decreasing the MMP presumably through the oxidation of critical thiols in membrane pumps and/or pores. The combination of these mechanisms and the ability of PQ to cause an increase of intracellular Ca^{2+} levels and ROS that can

further inactivate thiol-dependent Ca^{2+} pumps, which in turn, aggravates the hypercalcemia and thus DNA fragmentation.

The effect of PQ on apoptosis was also analyzed by phosphatidylserine translocation, a hallmark of early apoptosis. There are no significant changes observed in cells following a 6 hour exposure to 1.0, 2.0 or 5.0 mM PQ (Figure 10). A study by Cappelletti *et al.* (1998) demonstrated that A549 cells incubated with PQ (80 or 160 μM) for 24 hours showed no apoptotic features until their subsequent incubation in PQ-free medium, which resulted in a time-dependent appearance of apoptosis. Kang *et al* (2006), on the other hand, found that the incubation of PC12 cells (rat adrenal medulla pheochromocytoma cells) with 300 μM PQ for 24 hours resulted in a significant increase of apoptotic features assessed by annexin V binding and PI uptake.

Flow cytometric analysis of phosphatidylserine translocation was also assessed for an effect of LA treatment of PQ-challenged A549 cells. A study by Choi *et al.* (2009) found that the incubation of A549 cells with 0.5 or 1.0 mM LA for 24 hours resulted in an increase in cytochrome c levels. Cytochrome c is normally found between the inner and outer mitochondrial membranes where it functions as a part of the electron transport chain. When the mitochondrial membrane becomes depolarized, cytochrome c spills out into the cytosol and associates with Apaf-1 to form an apoptosome. The apoptosome attracts and activates pro-caspases 9 into active caspase 9 that triggers a cascade of events that leads to apoptotic cell death (Weinburg, 2007). Similar to PQ, 6 hour exposure to 0.1, 0.5 or 1.0 mM LA resulted in no significant change in apoptotic features indicated by

phosphatidylserine translocation (data not shown). Our studies also found no significant apoptotic features of LA treatment on PQ-challenged A549 cells assessed by phosphatidylserine translocation.

The results of the present study suggest that PQ administration resulted in cellular disturbances such as elevation of intracellular calcium, decreases in MMP and increases in DNA fragmentation 6 h post PQ-administration. It has been shown that chemicals that adversely affect cellular energy metabolism, Ca^{2+} homeostasis and redox state result in necrosis and/or apoptosis. The potentiating effects of LA in PQ-induced cellular changes are evidence to suggest that LA does not act as an antioxidant. Whether LA and/or its metabolite(s) prime the cells to undergo necrosis or apoptosis remains to be elucidated. Studies have shown that LA can induce apoptosis in cancer cells via caspase-dependent and independent apoptotic pathways regulated by increases in intracellular calcium concentrations (Choi *et al.*, 2009; Schweizer & Richter, 1996)

Conclusion

The results of this *in vitro* study have revealed that PQ and H₂O₂ exhibit a concentration- and time-dependent cytotoxicity in A549 cells. It has been shown that LA and/or DHLA are unable to directly scavenge H₂O₂, which was supported by our preliminary results where LA was unable to protect against a decrease of cell viability in H₂O₂-challenged A549 cells. PQ toxicity was found to occur primarily through the dysregulation of cellular metabolic activity resulting in cell death. PQ inhibits the transfer of electrons across complex I of the electron transport chain causing a decrease in ATP synthase activity, an increase in intracellular calcium levels, and an increase production of ROS. These three factors contribute to the dissipation of the mitochondrial membrane potential, an increase in DNA fragmentation and eventually cell death. No significant apoptotic features were discovered in this study, but literature would suggest there is a time-dependent appearance of apoptotic features after PQ-insult. Depending on the nature of oxidative stress and physiological circumstances, LA and/or DHLA can act as a pro- or anti-oxidant. LA delivered to A549 cells did not confer protection against PQ-induced cytotoxicity, instead exhibited a potentiating effect. These effects were reflected in the dissipation of the mitochondrial membrane potential and the enhancement of DNA fragmentation and intracellular calcium levels of PQ-challenged A549 cells. It is speculated that these potentiating adverse effects are a result of the oxidation of protein thiols, PARP-1 and AIF expression and ROS production. These results suggest that future studies are justified regarding the potentiation of oxidant-induced lung toxicity by redox active LA, taking into account the prooxidant properties of LA. Furthermore, *in vitro*

studies looking into the pharmacological use of LA in cancer chemotherapy should be considered.

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